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Sinclair and Dyes Inlets Toxicity Study: An Assessment of Copper Bioavailability and Toxicity in Surface Waters Adjacent to the Puget Sound Naval Shipyard and Intermediate Maintenance Facility

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ACRONYMS AND ABBREVIATIONS

ACR	Acute-Chronic Ratio
APDC	Ammonium Pyrrolidinedithiocarbamate
ASTM	American Society for Testing and Materials
AWQC	Ambient Water Quality Criteria
BLM	Biotic Ligand Model
CAS	Columbia Analytical Services, Kelso, WA
CCC	Criterion Continuous Concentration (chronic limit)
CMC	Criterion Maximum Concentration (acute limit)
CuCC	Copper Complexation Capacity
CuISE	Copper Ion-Specific Electrode
CWA	Clean Water Act
DDDC	Diethylammonium Diethyldithiocarbamate
DMR	Discharge Monitoring Report
DOC	Dissolved Organic Carbon
DRC	Dynamic Reaction Cell
EC50	Effect Concentration for 50^{th} percentile
ECx	Effect Concentration for x^{th} percentile
Ecology	Washington State Department of Ecology
ENVVEST	ENVironmental InVESTment
EPA	Environmental Protection Agency
ELAP	Environmental Laboratory Accreditation Program
FAV	Final Acute Value
FIAM	Free Ion Activity Model
FIAS	Flow Injection Analysis System
GC	Granite Canyon, Carmel, CA
GMAV	Genus Mean Acute Value
GLP	Good Laboratory Practices
GSIM	Gill Surface Interaction Model
HDPE	High Density Polyethylene
HNO_3	Nitric Acid
ICP-MS	Inductively Coupled Plasma-Mass Spectrometer
LOEC	Lowest Observed Effect Concentration
LW	Lab Water
NOAA	National Oceanographic and Atmospheric Administration
NOEC	No Observed Effect Concentration
NPDES	National Pollutant Discharge Elimination System
NWFSC	Northwest Fisheries Science Center, Seattle, WA
pCu	$-\log [Cu(II)_{aq}]$
PEMES-3	Perkin-Elmer Multi-Element Standard Solution
PSNS&IMF	Puget Sound Naval Shipyard & Intermediate Maintenance Facility, Bremerton, WA
Q-HNO_3	Reagent-Grade Nitric Acid
RT	Reference Toxicant
SIO	Scripps Institute of Oceanography, San Diego, CA
SMAV	Species Mean Acute Value
SOP	Standard Operating Procedure
SRM	Standard Reference Material

SSC Pacific	Space and Naval Warfare Systems Center Pacific, San Diego, CA
STGFAA	Stabilized Temperature Graphite Furnace Atomic Absorption
SWN	Suwanne River, FL
TOC	Total Organic Carbon
TSK	Trimmed Spearman Karber
TSS	Total Suspended Solids
U.S.	United States
WER	Water-Effect Ratio
WET	Whole Effluent Toxicity
WQC	Water Quality Criteria
WQS	Water Quality Standard

EXECUTIVE SUMMARY

A toxicity assessment was conducted to evaluate potential toxicity and bioavailability of copper (Cu) in surface water samples collected from locations in Sinclair and Dyes Inlets, adjacent to the Puget Sound Naval Shipyard & Intermediate Maintenance Facility (PSNS&IMF) in Bremerton, Washington. Ambient site water samples were collected in spring, winter, and late summer/fall and tested for toxicity to mussel (*Mytilus galloprovincialis*) embryos in 48-hour embryo-larval development tests using protocols recommended by the U.S. Environmental Protection Agency (EPA) for calculating water-effect ratios (WER). The ambient water samples from Sinclair and Dyes Inlets were not toxic to mussel embryos during the tests, and had dissolved copper concentrations (average 1.06 µg/L; range = 0.6 to 2.1 µg/L) that averaged three times lower than the ambient water quality criteria (AWQC) for continuous (3.1 µg/L [chronic limit]) and over four times lower than maximum (4.8 µg/L [acute limit]) exposure. Reduced normal survival of mussel embryos was observed in two samples from the late summer/fall sampling event, but the toxicity was attributed to the presence of very high concentrations (> 10^5 cells/L) of a toxic dinoflagellate, *Gymnodinium splendens*, rather than exposure to contaminants.

Copper additions to site and laboratory waters always resulted in toxic effects to developing mussel larvae. The measured copper concentration causing an effect in 50% of the test animals (EC50) in the site water toxicity tests was always higher than EC50s generated in laboratory water comparable to that used in AWQC development. As expected, total recoverable EC50 values were significantly correlated with total suspended solids, and dissolved EC50s were significantly correlated with dissolved organic carbon concentration. Final dissolved and total recoverable WERs of 1.41 and 1.63 were calculated, respectively, following the determination of no statistical differences among individual WERs across sampling seasons and among the sampling locations within a sampling event. These findings indicate that overall conditions within the Inlets were responsible for reducing the toxicity of copper to mussel embryos by a factor of 1.41, on a dissolved basis. Therefore, an adjustment of the national AWQC for dissolved copper by a factor of 1.41 would still provide the same level of protection intended by the U.S. EPA. Using this WER, acute and chronic site-specific dissolved copper criteria for Sinclair and Dyes Inlets, would be 6.8 and 4.4 µg/L, respectively.

Developmental tools that show promise as a means of predicting WERs using various rapidly obtained measurements were also evaluated in this study. Models based on the linkages between toxicity and dissolved organic carbon (DOC) concentration, free copper ion concentration (pCu_{tox}) and copper complexation capacity (CuCC), a chemical measure of bioavailability based on free copper measurements, correlated well with copper effect levels (EC50)(r^2 = 0.6 to > 0.7). Empirically derived (toxicity test-based) WERs from this study were within 5% of those predicted using these models. The development tools also allowed for the prediction of similar final WERs (range = 1.27 to 1.40) using larger sample sizes (n = 117 for DOC, n = 26 for pCu_{tox} and CuCC) than that used for the toxicity study (n = 13). The similarity between empirically derived and predicted WERs using the DOC model both supports the results of the toxicity study and helps validate these models as effective and less costly means of deriving site-specific criteria for copper in saltwater environments.

The very high sensitivity of *M. galloprovincialis* embryos to relatively low concentrations of dissolved copper makes it a relevant test endpoint on which to base a WER study. Recent studies indicating high copper sensitivity to salmonid endpoints (e.g., olfactory inhibition) were generally conducted in waters with characteristics appreciably different than those expected in Sinclair and Dyes Inlets. Samples of seawater obtained from the Mukilteo Field Station were analyzed for Cu, DOC, TSS, and mussel embryo toxicity to provide data on Cu bioavailability in the same site water

used for the study of the effects on Cu on sublethal olfactory impairment in Chinook smolts in salt water (D.H. Baldwin, Northwest Fisheries Science Center, Seattle, WA, personal communication). The seawater from the Mukilteo Field Station had low dissolved Cu (average 0.15 µg/L; range 0.1 – 0.19 µg/L), DOC (average 1.5 mg/L, range 1.4 – 1.9 mg/L), and TSS (average 13 mg/L, range 6 – 30 mg/L) concentrations. The seawater from the Mukilteo Field Station had very little binding capacity for Cu, and consequently, mussel embryos were very sensitive to Cu exposure, resulting in mussel embryo Normal Survival EC50s that ranged from 5.2 to 5.87 µg/L and NOECs and LOECs of 4.1 and 5.8 µg/L dissolved Cu, respectively. As expected, the NOEC and LOEC for seawater samples from the Mukilteo Field Station were above the chronic and acute water quality standards for dissolved Cu. Use of the Biotic Ligand Model to normalize toxic concentrations based on expected site-specific conditions (e.g., hardness, DOC concentrations) indicate that olfactory impairment would be adequately protected under a site-specific criterion based on the *M. galloprovincialis* results.

Based on the current regulatory acceptance of the WER method and the availability of a site-specific, toxicity-derived WER determined by this study, site-specific criteria for Cu discharges in Sinclair and Dyes Inlets should be considered in developing National Pollutant Discharge Elimination System (NPDES) permits in the local region.

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INTRODUCTION

This document describes the results from an assessment of both ambient toxicity and copper (Cu) bioavailability, using toxicity tests with mussel embryos, in surface waters from Sinclair and Dyes Inlets, adjacent to the Puget Sound Naval Shipyard & Intermediate Maintenance Facility (PSNS&IMF), in Bremerton, Washington. Copper is a contaminant of concern in estuarine and coastal environments because of its potential to impact the biota at very low concentrations. Copper discharges from the Shipyard are regulated under a National Pollutant Discharge and Elimination System (NPDES) permit issued by the U.S. Environmental Protection Agency (U.S. EPA, 1994b). Sources of copper to Sinclair and Dyes Inlets include the shipyard's industrial discharges (U.S. EPA, 1994b), storm water and nonpoint-source runoff (Brandenberger et al., 2005, 2006), and leaching from copper-based ship hull coatings (Johnson et al., 1998a, 1998b).

The national ambient water quality criterion (WQC) for copper in saltwater environments recommended by the U.S. EPA is based on the Criterion Maximum Concentration (CMC) of 4.8 µg dissolved Cu/L which "…is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed briefly without resulting in an unacceptable effect" (acute exposure), and the Criterion Continuous Concentration (CCC) of 3.1 µg dissolved Cu/L which "… is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect" (chronic exposure, U.S. EPA, 2006). These criteria "…are intended to be protective of the vast majority of the aquatic communities in the United States (U.S. EPA, 2006) and excursions above the criteria put sensitive aquatic organisms at risk. The above criteria are applied to all surface waters as Washington state water quality standards (WQS) by the Department of Ecology (Ecology), however, the department may revise the criteria on a statewide or water body-specific basis as needed to protect aquatic life occurring in waters of the state and to increase the technical accuracy of the criteria being applied (Ecology, 2003). Many studies have shown that site-specific physical and chemical characteristics (e.g., dissolved organic carbon, suspended solids, natural ligands, etc.) of seawater influence the bioavailability and toxicity of metals to aquatic organisms (Stauber et al., 2000, Knezovich, Harrison, and Tucker, 2001; Eriksen, Mackey, van Dam, and Nowak, 2001; Lorenzo, Nieto, and Beiras, 2002; Rosen, Rivera-Duarte, Kear-Padilla, and Chadwick, 2005; Arnold, Cotsifas, and Corneillie, 2006). Therefore, individual water bodies will differ in their potential to buffer against metal toxicity, and development of site-specific criteria may be warranted.

The approach recommended by U.S. EPA (U.S. EPA, 1994a, U.S. EPA, 2001) and Ecology (Ecology, 2006) to assess site-specific metal bioavailability is to spike water samples from the site with different concentrations of copper in the laboratory and compare the resulting median lethal or effects concentrations (LC50 or EC50) in site water to those observed in concurrent exposures using water similar to that used in WQC derivation (e.g., laboratory water). In this study, we followed U.S. EPA's guidance (U.S. EPA, 1994a, 2001) to determine the site-specific WQC for copper by calculating a water effect ratio (WER) for water samples collected from Sinclair and Dyes Inlets, Washington. In short, a WER is calculated by dividing the EC50 calculated in site water by the EC50 in laboratory water. The ratio, which is typically greater than 1 in bays and estuaries, is then multiplied by the national WQC to determine the site-specific criterion. The rationale for deriving site-specific WQC is to provide flexibility in establishing discharge limits under the NPDES while still providing the same level of protection intended in the U.S. EPA's WQC derivation guidelines (U.S. EPA, 1985).

Numerous studies throughout the nation have examined the application of WERs as a means of determining the site-specific toxicity of copper (Gauthier et. al., 1995, Rosen et al., 2005). In the

marine environment, WER studies have generally resulted in an adjustment of the national criterion by a factor of about 2. In a study conducted for naval bases in the Hampton Roads, Virginia (USA) area, WER tests with a marine copepod (*Acartia tonsa*) resulted in total recoverable and dissolved WERs of 2.30 and 1.76, respectively (CH2M Hill, 2000). A New York Harbor (New York, New York, USA) WER study resulted in a dissolved WER of 1.5, using a combination of three species, including *Mytilus edulis* as well as the sea urchin *Arbacia punctulata* (U.S. EPA, 1994c). San Francisco Bay (California, USA) has been the focus of several WER studies. A bay-wide total recoverable WER of 1.7 was obtained in 1991 using toxicity tests with embryos of the Pacific oyster (*Crassostrea gigas*), while a subsequent study of South San Francisco Bay that employed *M. galloprovincialis* embryos resulted in total and dissolved WERs of 3.66 and 2.77, respectively (City of San Jose, 1998). In a study conducted for San Diego Bay, California, dissolved WERs were estimated at 1.54-1.67, while total recoverable WERs were estimated at 2.07 to 2.27 (Rosen et al., 2005).

In many of these cases, the magnitude of the WER has been linked to the concentration of total suspended solids (TSS) and/or dissolved organic carbon (DOC) concentrations at the sites. In particular, DOC concentration appears to be able to reliably predict mussel embryo EC50s exposed to dissolved copper (Arnold, 2005; Arnold, Cotsifas, and Corneillie, 2006). In the recent update of copper criteria recommended for fresh water (U.S. EPA, 2003), EPA used the biotic ligand model (BLM) (Di Toro et al., 2001) to calculate the toxic fraction of dissolved copper present as free ionic copper present in natural waters (U.S. EPA, 2003). The BLM uses a variety of physico-chemical input parameters (e.g., pH, hardness, DOC) to predict toxicity. The BLM is based on the concept that toxicity occurs when the metal–biotic ligand complex reaches a critical concentration. For fish, the biotic ligand is either known or suspected to be the sodium or calcium channel proteins in the gill surface that regulate the ionic composition of the blood (DiToro, et al., 2001; Santore et al., 2001). For other organisms, it is hypothesized that biotic ligands exist and that mortality can be modeled in a similar way. The biotic ligand interacts with the metal ions in solution. The amount of metal that binds is determined by a competition for metal ions between the biotic ligand and the other complexing ligands, particularly dissolved organic matter (DOM), and the competition for the biotic ligand between the toxic metal ion and the other metal ions in solution. The model is a generalization of the free ion activity model (FIAM), which indicates that the free metal ion is the best predictor of toxicity. Currently, the amount of DOC and other complexing ligands are expected to play an important role in the development of a saltwater Biotic Ligand Model (BLM), which may be used as an alternative to WER studies for the development site-specific criteria (Arnold, 2005).

Because of the expense and time involved in conducting the extensive toxicity testing and associated chemical analyses associated with a WER study, alternative means of predicting copper bioavailability are desired. Promising methods are the DOC-model and BLM introduced above. Another method, based on a similar philosophy, is to directly measure the complexation capacity of the site water. Copper complexation capacity (CuCC) is a chemical measurement determined with a copper ion selective electrode (CuISE), in response to systematic addition of copper in ambient water (Rivera-Duarte and Zirino, 2004). The response of the CuISE is indicative of the concentration of aqueous free copper ion ($Cu(II)_{aq}$), which has been shown to be a better predictor of toxicity than total or dissolved measurements (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Eriksen et al,, 2001; Rivera-Duarte et al., 2004), and is the basis of the BLM.

BACKGROUND

Sinclair and Dyes Inlets, Washington, were listed on the 1998 303(d) list of impaired waters because of fecal coliform (FC) contamination in the marine waters and metals and organic contaminants in bottom sediments and fish tissues (Ecology, 1998). The PSNS&IMF (Figure 1), Washington Department of Ecology (Ecology), U.S. Environmental Protection Agency (U.S. EPA) and local stakeholders are working together on Project ENVVEST (an acronym for ENVironmental InVESTment) to address contamination issues and develop water cleanup plans for the watershed (U.S. Navy, EPA, and Ecology, 2000; ENVVEST, 2006). Ambient water quality monitoring was conducted by Project ENVVEST to assess the impact of storm event runoff on the water quality of the Inlets, evaluate the potential ecological significance of discharges in the Inlets, and provide data that can be used to support the development of an alternative regulatory strategy for the Shipyard (Johnston et al., 2005). As part of the ambient monitoring, samples were also collected for toxicity evaluation following established protocols developed for evaluating site-specific water quality criteria with particular emphasis on evaluating processes that affect the bioavailability of copper, such as complexation capacity (Rivera-Duarte et al., 2005) and the BLM (Niyogi and Wood, 2004; U.S. EPA, 2003; WEF, 2004a,b). The results of the toxicity evaluations are the subject of this report.

Figure 1. The location of PSNS & IMF adjacent to Sinclair and Dyes Inlets, Washington.

Dry dock and storm water discharges from the Shipyard to Sinclair Inlet require NPDES permits issued by the U.S. EPA. The current dry dock permit has average monthly and maximum daily total recoverable copper concentration limits. The average monthly concentration is 19 ppb and the maximum daily concentration is 33 ppb. The current NPDES permit contains the requirement to monitor storm water, but does not contain numerical limits (U.S. EPA, 1994b). In addition, the permit also specifies loading limits for the dry dock discharges expressed in pounds per day. Although acute and chronic water quality criteria are not exceeded in Sinclair Inlet (Katz et al., 2004), the potential for adding to elevated copper levels in sediments adjacent to the Shipyard is a concern.

The objective of this study was to assess the assimilative capacity of copper in surface waters at sites adjacent to PSNS&IMF and other locations in Sinclair and Dyes Inlets. In general, water bodies have a greater capacity to reduce a metal's bioavailability than the laboratory water typically used for derivation of national ambient WQC, upon which discharge permits are based. This is because laboratory water tends to be low in metal-binding particulate matter and dissolved organic matter compared to most ambient waters (U.S. EPA, 1994a,c). These differences in bioavailability are accounted for by U.S. EPA's water effect ratio (WER) procedure in which metal-spiked site and laboratory waters are evaluated for toxicity in side-by-side exposures. The site water median effective concentration (EC50) is then divided by the lab water EC50, resulting in a multiplier that can be used to adjust the national WQC. This work follows the "Method 2 WER Guidance" for determining a WER for a large water body outside the vicinity of plumes (U.S. EPA, 1994a). The work also drew upon updated guidance associated with the streamlined WER procedure for copper (U.S. EPA, 2001). WERs were calculated to characterize the differences in bioavailability between several locations in Sinclair and Dyes Inlets over different seasons, and in laboratory water. Method 2 was designed to develop chronic WERs, but because the test endpoint (*Mytilus* embryo-larval development success) is above the CMC, the WERs obtained in the study can potentially be used to adjust both the national acute and chronic saltwater criteria for copper (U.S. EPA, 1994a; City of San Jose, 1998).

Copper complexation capacity (CuCC) is a chemical measurement that could be used as a surrogate for the types of toxicity tests described above. CuCC is defined as the capacity of ambient water to assimilate inputs of copper without associated adverse effects upon aquatic organisms. It is measured with a copper ion selective electrode (CuISE), in response to systematic addition of copper in ambient water. The response of the CuISE is indicative of the concentration of aqueous free copper ion ($Cu(II)_{aq}$) in solution, which according to the free-ion model (Buffle, Altman, Filella, and Tessier, 1990), and substantiated by experimental evidence (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Campbell, 1995, Ericksen et al, 2001; Rivera-Duarte et al., 2004), is the fraction of copper that is available to organisms, making it a better predictor of its potential toxicity than either the total or dissolved copper concentrations. Therefore, CuCC is a chemical measurement indicative of the toxicity of the water to organisms and is very much related to the results from toxicity tests (larval development EC50).

METHODS

STUDY GOALS

The goals of this study were to (1) assess the potential for ambient toxicity, and (2) determine relative copper bioavailability in surface waters adjacent to PSNS&IMF. These goals were achieved through laboratory-based toxicity exposures using sensitive endpoints following EPA's water effect ratio procedure for developing site-specific water quality criteria (U.S. EPA, 1994a: U.S. EPA, 2001; Ecology, 2006), as well as via chemical measurements to determine the complexation capacity at the site. To address the potential for variability over space and time, as many as five sites were evaluated for three sampling events conducted during different seasons (Spring 2004, Winter 2005, and Summer/Fall 2005) for the toxicity study. As many as 10 sites were evaluated over 5 sampling events for CuCC measurements.

STUDY SITE

Ambient Sampling Locations

Sample locations evaluated for toxicity and CuCC are listed in Table 1 and shown geographically in Figure 2. The stations were collocated with the sampling sites planned for the fecal coliform model verification study (Johnston et al., 2004). Additionally, the stations were sampled for dissolved and particulate metals, total suspended solids, total organic carbon, dissolved organic carbon, salinity, alkalinity, and pH (Johnston et al., 2004). These sites were assumed to be representative of ambient conditions within the Inlets during the sampling periods.

Table 1. Stations sampled for copper toxicity (CuTOX) and copper complexation capacity (CuCC) analyses.

	Sampling Event							
Station	3/31/2004		2/9/2005		3/2/2005	6/20/2005	9/27/2005	
	CuTOX	CuCC	CuTOX	CuCC	CuCC	CuCC	CuTOX	CuCC
M3.1	X	X	X	X	X	X	X	X
M3.3	X	X						
P3	X	X	X	X	X	X	X	X
SN12			X	X	X	X	X	X
M4			X	X	X	X	X	X
M6			X	X	X	X	X	X
M3.2				X		X		X
DY01				X		X		X
P1				X		X		X
P2				X		X		X
BJ-EST				X		X		X

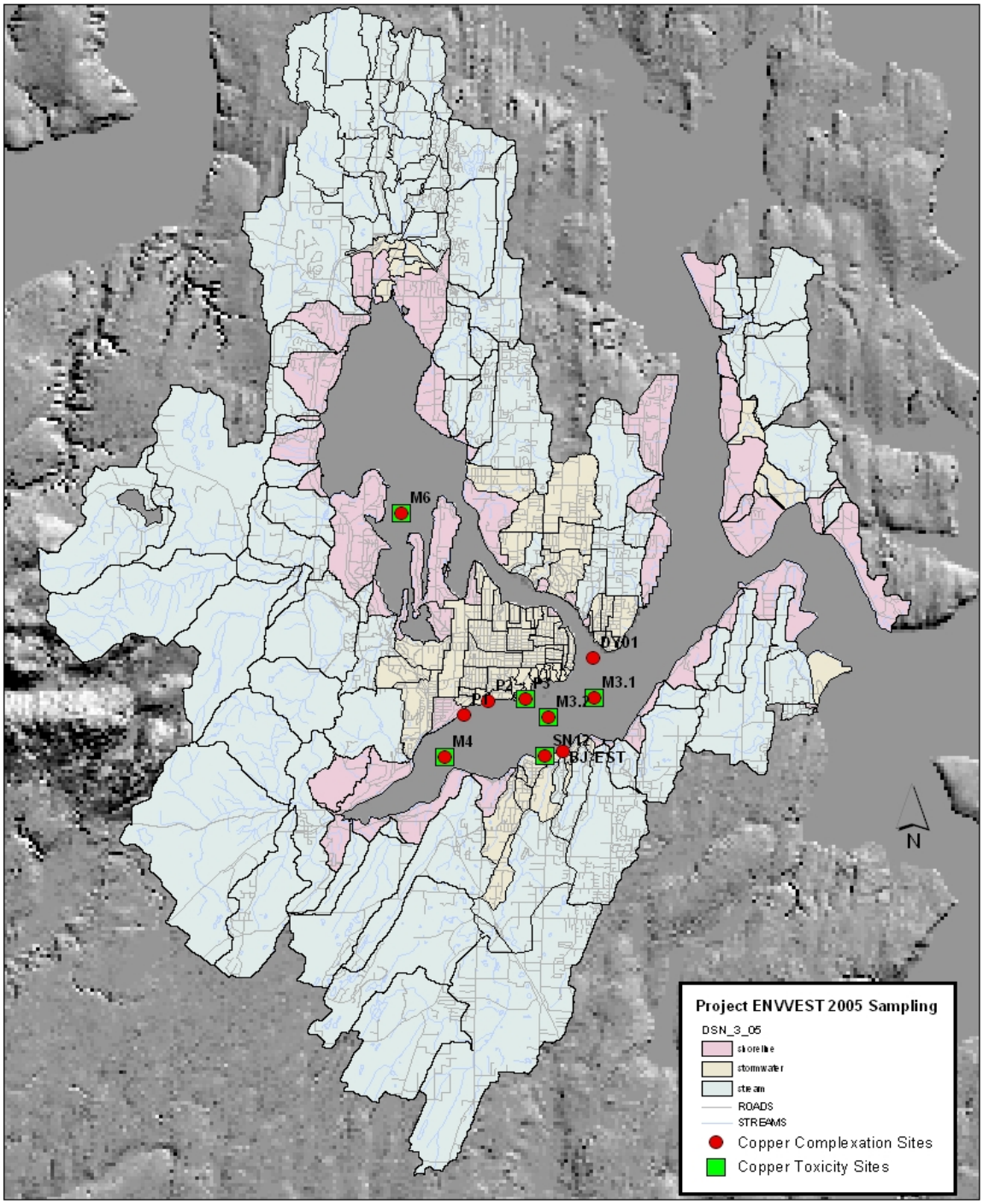

Figure 2. Marine sampling stations near the PSNS&IMF. The stations with green squares were sampled for the toxicity testing, while the red circles indicate stations that were measured for copper complexation capacity.

Sample Collection and Storage

Site water was collected from the water surface (depth of ~1 m) using clean techniques (U.S. EPA, 1995c) for each of the sampling events. Prior to sampling, new pre-cleaned 1-L HDPE bottles were thoroughly rinsed with ultra pure (E-pure™) water. Samples were shipped on ice overnight to SPAWAR Systems Center Pacific (SSC Pacific). Upon arrival, samples were immediately evaluated for condition and water quality parameters, including arrival temperature (Appendix A). If necessary, samples were stored at approximately 4 °C upon arrival in the laboratory, but, in general, toxicity test setup commenced immediately upon arrival. Holding time of samples for WER studies is limited to 96 h following sample collection (U.S. EPA, 2001). Additional samples were collected for copper analyses, as well as total suspended solids (TSS) and dissolved organic carbon (DOC), and their handling is described in their respective sections. Upon arrival in the laboratory, CuCC samples were immediately frozen for later analysis.

TOXICITY ASSESSMENT

Site and Laboratory Water Preparation

Analyses of site water under the microscope indicated the presence of live zooplankton and phytoplankton for most samples, but predation on mussel embryos was not expected. Therefore, to best preserve sample integrity, samples were tested without any pre-sieving. Site water salinity was generally 29 to 30 ‰, within range of the test protocol and that tolerated by the test species. Therefore, no salinity adjustment was made to the samples.

Laboratory water consisted of filtered (0.45 µm) coastal seawater from the research pier at Scripps Institute of Oceanography (SIO) in La Jolla, California. SIO seawater is used routinely as control (reference) and dilution water by several bioassay laboratories in the San Diego region. SIO seawater is relatively free of contamination, low in TSS, and contains low levels of DOC, which are characteristics of "laboratory" water used for WQC development (U.S. EPA, 1985). To achieve testing salinities comparable to the site water from Sinclair and Dyes Inlets, the SIO seawater (lab water), which has a natural salinity of approximately 34 ‰, was diluted to the site water salinity (29 ‰) with E-pure™ (18-MΩ) water. All water quality measurements and chemical analyses for the laboratory water were made on the diluted preparations.

Test Species

Toxicity testing was conducted with embryos of the Mediterranean mussel, *Mytilus galloprovincialis*. This species and life stage is relevant because embryogenesis in *Mytilus sp.* is impacted by copper at very low concentrations (GMAV = 9.6 ppb; U.S. EPA, 1995b), and *M. galloprovincialis* is present as a commercially important species in the Puget Sound area (Taylor Shellfish Farms, 2004). The 48-hour embryo-larval development endpoint for *Mytilus sp.* is the driver of the current saltwater ambient WQC of 4.8 (acute) and 3.1 (chronic) µg dissolved Cu/L, with the GMAV falling below the final acute value (FAV) derived from 26 different species (U.S. EPA, 1995b; Figure 3).

The test endpoint is recommended by the EPA for use in WER studies (U.S. EPA, 1994a). *M. galloprovincialis* has also been used as a test species for caged mussel deployments in Sinclair Inlet during the installation restoration investigations conducted in 1994 (URS, 2001) and 2005 (Johnston et al., 2004; Salazar et al., 2006). Development of the saltwater Biotic Ligand Model (BLM) for copper has also focused specifically on this species and toxicity test endpoint. *M. galloprovincialis* used in this study were obtained from Carlsbad Aquafarm, Carlsbad, California.

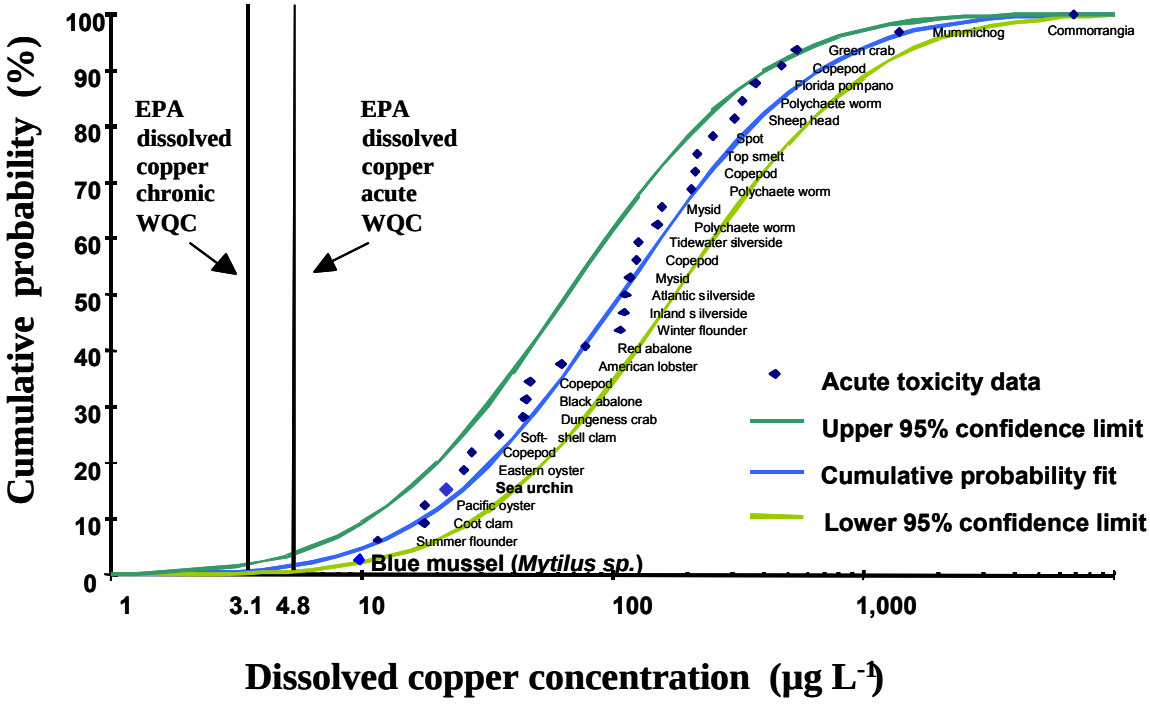

Figure 3. Species sensitivity distribution for copper in saltwater (U.S. EPA, 1995b).

Toxicity Tests

Toxicity tests were conducted following ASTM and U.S. EPA guidance for whole effluent toxicity (WET) testing (ASTM, 1999; U.S. EPA, 1995a) and for determining WERs (U.S. EPA, 1994a, U.S. EPA, 2001). The toxicity tests were performed at the SSC Pacific Environmental Sciences Bioassay Laboratory (ESBL), San Diego, California. The ESBL maintains laboratory certifications for bio-assays from the Washington State Department of Ecology and the State of California Laboratory Accreditation Programs, employs qualified toxicologists, conducts external and internal audits, and maintains up-to-date standard operating procedures (SOPs) and good laboratory practices (GLP).

Toxicity testing consisted of the following procedures. Site and laboratory water samples were spiked with as many as eight nominal copper concentrations, including 0, 2.9, 4.1, 5.9, 8.4, 12, 17.2, 24, and 50 µg/L. Copper stock solutions were made from reagent grade copper sulfate salts and confirmed by stabilized temperature graphite furnace atomic absorption (STGFAA) spectroscopy prior to use. The same stock solution was used for laboratory waters, site waters, and any associated reference toxicant tests. Test concentrations were prepared separately in acid-cleaned and seawater leached 125-mL Erlenmeyer flasks. From each flask, 10 mL was distributed to each of five new, seawater-conditioned, glass 20-mL scintillation vials for the bioassay. A sixth replicate for at least one test concentration per sample was also included in the test, and used for quantification of total recoverable and dissolved copper by STGFAA at the end of the test, in order to account for any change in copper concentration compared to initial concentrations. An equilibration period of 3 to 5 h was allowed following copper additions prior to addition of embryos.

During test set up, 20 mL of each test solution was also dispensed into an acid-cleaned, high-density polyethylene (HDPE) scintillation vial. Within 24 h, 10 mL of each of these samples was filtered using clean techniques (see Copper Measurements section) using acid-cleaned 0.45-µm polycarbonate membrane filters, into another HDPE scintillation vial. The remaining unfiltered sample and the filtered samples were then immediately acidified with concentrated (15 N) ULTREX nitric acid until analysis. The sixth test replicate, for chemical analysis, was handled in the same manner at the end of the toxicity exposures.

Specimens of *M. galloprovincialis* were obtained from Carlsbad Aquafarm, Carlsbad, CA on the same day tests were initiated. Mussels were induced to spawn by thermal shock (raising the temperature by about 10 °C from ambient). Within 4 h of fertilization, approximately 200 embryos at or beyond the two-cell stage were added to each test vial. Vials were then incubated at 15 ±1 °C for 48 h under a 16-h light: 8-h dark photo period. Water quality (pH, temperature, dissolved oxygen, salinity) was recorded at test initiation for all tests and daily for the 2/9/05 and 9/27/05 events. A summary of the target test conditions and test acceptability criteria are provided in Appendix B. Water quality measurements are summarized in Appendix C.

After 48 h of exposure, normally developing mussel embryos will achieve the prodissoconch I stage, characterized by a straight-hinged D-shaped larval shell. Two different endpoints were used to assess larval development at the end of the test: (1) normal survival (number of surviving embryos exhibiting normal development), and (2) proportion normal (proportion of surviving embryos exhibiting normal development). The normal survival endpoint was used for EC50 calculations because it is more comprehensive, combining both survival and normal development. Larvae were evaluated with the aid of an inverted compound microscope at 40 to 60x magnification.

Data Analysis

EC50s were calculated from normal survival data with ToxCalc™ version 5.0, using the Maximum Likelihood Probit or the Trimmed Spearman Karber (TSK) methods. Comparison of EC50s determined with Probit and TSK with those exclusively calculated with computational linear interpolation revealed negligible differences. EC50 and WER values were calculated from nominal, total recoverable, and dissolved copper concentrations for each test. WERs for each site water sample were calculated by dividing the site water EC50 by the associated lab water EC50. One-way ANOVA or t-tests were used to determine if WERs were significantly different among the sampling events, and where possible among the individual stations across events, at a significance level of 0.05. No observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) were obtained from hypothesis testing following arc-sine square root transformations of the toxicity data, and verification of normal distribution of data and homogeneity of variances using Shapiro-Wilkes and Bartlett's tests, respectively.

The potential for ambient toxicity was assessed by comparison of development success in the controls for each test (site water with no added copper) with test acceptability criteria for control performance. Control development in the site waters was also compared with that in the lab water using paired t-tests ($\alpha = 0.05$). Linear regression analysis was used to assess the relationship between EC50 and TSS, DOC, and copper complexation capacity (CuCC). One-way analysis of variance ($\alpha = 0.05$) was used to detect differences among WER values.

Quality Assurance

The toxicity testing was conducted and evaluated using quality assurance (QA) procedures in accordance with the SSC Pacific ESBL QA Plan, which is based on applicable protocols and guidance documents. These procedures include all aspects of testing, including the source, handling,

condition, receipt, and proper storage of samples and test organisms, as well as the appropriate calibration and maintenance of instruments and equipment. All data generated by the laboratory were evaluated for completeness and accuracy. Appropriate laboratory controls were conducted with each test, and were required to meet specific test acceptability criteria. For the mussel test, ≥ 70% normal survival in the controls is required for the test to be acceptable. In addition, reference toxicant tests were conducted with each test, as a measure of the laboratory's performance and test batch sensitivity. Reference toxicant EC50 values were required to be within two standard deviations of the laboratory's running mean. Minor excursions of targeted water quality objectives (Appendix B) during the tests were evaluated for their impact on the tests on a case-by-case basis. Excursions in temperature and salinity of less than 1 ºC or 1 ‰, respectively, were considered inconsequential.

COPPER MEASUREMENTS

Concurrent with the toxicity samples, additional water samples were collected for measurement of total recoverable, dissolved, and free copper ion concentrations, as well as copper complexation capacity (CuCC). Total recoverable and dissolved copper concentrations were used to support the toxicity assessment and WER calculations by allowing precise EC50 determination for each form of the metal. Free copper ion concentrations were used for determining the CuCC.

Total recoverable and dissolved copper

Sampling protocols for the ambient waters followed EPA Method 1669, EPA's Trace Metals Sampling Technique (U.S. EPA, 1995c). These include the use of acid-cleaned apparatus and materials made of polyethylene, and "clean hands/dirty hands" techniques. Preservation, handling, and analysis of the samples were conducted in class-100 trace metal clean working areas. Enough ULTREX grade nitric acid was added to the samples to decrease the pH to less than 2. Copper concentrations were measured by STGFAA spectroscopy either by direct injection (for spiked samples) or after liquid–liquid preconcentration with dithiocarbamates (for ambient samples) following Bruland, Coale, and Mart (1985). The standard reference material (SRM) CASS4 (coastal seawater) from the National Research Council of Canada was used to quantify the recovery of the preconcentration, and SRM 1643d (trace metals in water) of the National Bureau of Standards was used to evaluate the precision and accuracy of the STGFAA analysis. As recommended by the WER guidance (U.S. EPA, 1994a), a single test concentration near the expected EC50 was analyzed both at the beginning and end of the exposure by STGFAA to ensure that exposure did not drop by more than 50% due to metal loss.

Table 2. Detection limits for copper analysis.

		Method				
Parameter	Lab	PreConc.	Detection	Reporting Limit (µg/L)	Detection Limit (µg/L)	Blank (µg/L)
Total Cu	BMSL	Fe/Pd APDC	ICP-MS	0.13	0.0417	0.0874
Dissolved Cu	BMSL	Fe/Pd APDC	ICP-MS	0.13	0.0417	0.0874
Total Cu (Ambient)	SSC Pacific	APDC/DDDC	STGFAA	0.032	0.010	0.003
Total & Dissolved Cu (Spiked samples)	SSC Pacific	N/A (1N HNO_3 Dilution)	STGFAA	0.84	0.25	0.028

APDC - ammonium pyrrolidinedithiocarbamate
DDDC – diethylammonium diethyldithiocarbamate

Free copper ion and copper complexation capacity

The concentration of the free aqueous copper ion ($[Cu(II)_{aq}]$) was measured with an Orion 94-29 Cu(II) ion selective electrode (Cu-ISE), following procedures used by Zirino *et al* (1998), and Cu-CC was measured as detailed in Rivera-Duarte and Zirino (2004); however, a brief description of the procedures is provided here. Both measurements were made in a dark, class-100 working station, with constant stirring at 25 ±0.1°C, by the electrode potential (mV) between a Cu-ISE and an Orion Ag/AgCl double-junction reference electrode. The electrodes were calibrated with seawater Cu-activity buffers made with 2×10^{-4} M Cu in filtered (0.45 µm) seawater and either 1×10^{-3} M ethylenediamine or 1×10^{-3} M glycine (Belli and Zirino, 1993, Zirino et al., 1998). Since $[Cu(II)_{aq}]$ in each buffer was calculated with a specific ion-interaction model for the measured pH and the concentrations of major ions (Belli and Zirino, 1993), the calibrated response of the Cu-ISE is reported as the pCu (i.e., $-\log [Cu(II)_{aq}]$) of the solution.

The change in the response of the Cu-ISE during a titration with copper was used for the measurement of the Cu-CC (Rivera-Duarte and Zirino,, 2004). The titrations were performed with a TTT 85 Titrator and an ABU 80 Autoburette, both from Radiometer Copenhagen, connected to a personal computer for continuous automatic recording of the data. First, the electrodes were calibrated and then allowed to equilibrate overnight in an aliquot of the seawater sample. The next day, an aliquot of 250 to 300 g of fresh seawater sample was weighed into a Teflon® beaker, and the electrodes were allowed to equilibrate in it for several minutes before starting the titration. Once the potential stabilized to within 0.1 mV sec^{-1}, the titration proceeded automatically by additions of 10 µL each and was completed after 99 mL of the titrant was added. The titrant was made with 200 µL of 1000 ±3 µg mL^{-1} High Purity Copper Standard added to 1L of 18-MΩ water containing 32-g NaCl. Cu-CC was estimated from the inflection point of the resulting titration curve using a MATLAB® routine (Rivera-Duarte and Zirino, 2004).

DOC AND TSS MEASUREMENTS

Dissolved organic carbon (DOC) and total suspended solids (TSS) samples were collected concurrently with the site and laboratory water sampling for the toxicity tests. DOC samples were kept on ice and analyzed within 24 h of arrival at the analytical laboratory, according to EPA Method 415.1. The reporting limit for DOC was 0.5 mg/L. The TSS samples were analyzed according to EPA Method 160.2. The reporting limit for TSS was 0.5 mg/L, except for the 2/9/2005 sampling event, for which it was 5 mg/L.

RESULTS AND DISCUSSION

TOXICITY TEST ACCEPTABILITY AND QUALITY ASSURANCE

Samples were collected on 3/31/2004 (Spring), 2/9/2005 (Winter), and 9/27/2005 (Summer/Fall), comprising a total of 13 site water tests and 3 laboratory water tests. All three sampling events resulted in successful toxicity tests, with only a few minor deviations from targeted test conditions and test acceptability criteria (Appendix B). All reference toxicant tests resulted in EC50 values that were within two standard deviations of the testing laboratory's running mean, according to current control charts at the SSC Pacific Environmental Sciences Bioassay Laboratory (ESBL). The laboratory water served as the reference toxicant test in all cases. An additional copper reference toxicant test at a salinity of 34 psu was also conducted for the 3/31/2004 event, and this test also fell within normal control chart variability. Performance in the laboratory water controls (e.g., ambient laboratory water) was >70% normal survival for all three events (Table 3). In addition, all laboratory and site water samples exceeded 90% normal development of surviving controls (Table 3). The normal survival criterion of ≥70% was met by all but one of the site water samples, M4 from the 9/27/05 event. Normal survival in this sample was only 59%. This sample contained a very high density of toxic dinoflagellates (*Gymnodinium splendens*), but for reasons that will be elaborated upon, it was believed that the bioassay results from M4 were not compromised, therefore, they are included in the final data set.

Tests associated with all sampling events were initiated well within the 96 h holding time requirement for WER studies (U.S. EPA, 2001). Arrival temperature was exceeded slightly for the 9/27/05 event, with samples arriving at a range of 9.6 to 13.3 °C (Appendix A). Because of the short shipping time, testing temperature (15 °C) above the arrival temperature, and immediate processing of samples, it was concluded that this deviation was not of concern.

The pH and dissolved oxygen concentration were within targeted ranges for all measurements (Appendix C). Test temperature was within range for all tests, except a few for the 3/31/04 event, where excursions of < 0.3 °C were observed (Appendix C). A few salinity measurements were < 0.5 psu outside of the targeted range for the 2/9/05 event. Analysis of the data indicated that these minor exceedances were not significant to the data quality.

AMBIENT TOXICITY

Larval development in the unspiked (ambient) laboratory and site water samples was evaluated using two endpoints: proportion normal and normal survival. The proportion normal endpoint is defined as the number of normal straight-hinged, D-shaped, larvae relative to the total number of larvae (normal and abnormal) counted in a vial at the conclusion of the test. Of the surviving larvae, at least 70 or 90% must achieve normal shell development in the controls to meet test acceptability criteria, according to the ASTM (1999) and U.S. EPA (1995a) test methods (Appendix B), respectively. Proportion normal ranged from 94 to 99%, and no significant differences were observed when site waters were compared to lab waters, suggesting an absence of toxicity in all cases based on this endpoint.

Table 3. Control performance based on two different endpoints from toxicity tests performed with mussel (*Mytilus galloprovincialis*) embryos in laboratory water (LW) and site waters collected from Sinclair Inlet (M3.1, M3.3, M4, P3, SN12) and Dyes Inlet (M6), Washington. Controls are required to be above 70% for both endpoints to meet test acceptability criteria. Significant differences (α = 0.05) between the LW and site waters are highlighted in bold.

Sampling Date	Sample ID	Proportion Normal (%)		% Normal Survival			
		Mean	S.D.	Mean	S.D.	*p*	% of LW
3/31/2004	LW 98.	2	1.5	98	3.9	n/a	100
	M3.1	98.5	0.4	93	7.8	0.162	95
	M3.3	97.8	1.1	87	10.8	0.406	89
	P3	97.8	2.3	92	9.1	0.178	94
2/9/2005	LW 97.	7	1.0	100	16.5	n/a	100
	SN12	98.8	0.5	100	5.6	0.474	101
	M3.1	98.3	0.6	103	8.5	0.340	104
	M4	99.0	0.4	99	7.3	0.441	99
	P3	97.2	1.1	94	8.4	0.269	95
	M6	98.9	0.7	97	3.8	0.369	97
9/27/2005	LW 95.	3	0.4	89	6.2	n/a	100
	SN12	96.5	1.9	93	6.9	0.161	105
	M3.1	94.8	2.2	86	7.0	0.257	97
	M4	94.2	2.8	59	6.7	**<0.001**	66
	P3	94.9	2.0	85	8.9	0.260	96
	M6	97.5	1.4	81	6.6	**0.047**	91

In comparison, the normal survival endpoint measures the percentage of normally developed D-shaped larvae observed at the end of the test relative to the initial number of embryos added to the test vial, as determined from initial density vials preserved shortly after test initiation. Normal survival is generally considered a more comprehensive endpoint, as it considers both survival and normal larval development success. The control test acceptability criterion used in this study for normal survival is ≥70% (ASTM, 1999; Appendix B). All unspiked laboratory waters met the minimum criterion, with normal survival ranging from 89% (9/27/2005) to 100% (2/11/2005) (Table 3). In nearly all cases, the site waters also met this criterion (range = 87 to 103%, except for two samples) and were statistically indistinguishable from the lab water controls (Table 3). For the 9/27/2005 event, however, one sample (M4) produced control normal survival of only 59%. Furthermore, although M6 exceeded the 70% criterion with 81% normal survival, it too was statistically different from the controls in the lab water associated with that event (Table 3).

The relatively low performance of the unspiked controls, based on the normal survival endpoint, for M4 and M6 from the 9/27/05 event, however, is unlikely associated with contaminants of potential concern. Rather, both of these samples contained high concentrations of the autotrophic dinoflatellate *Gymnodinium splendens* (also known as *Gymnodinium sanguineum*) (Figure 4), which resulted in a very obvious brown hue to these samples. *G. splendens* is a vertically migrating cosmopolitan species that has been observed previously in both Sinclair and Dyes Inlets during late summer and fall months (Ecology, 1995). There are several ways that the dinoflagellates may have impacted the developing mussel embryos. First, *Gymnodinium spp.* are known to produce a neurotoxin known as saxitoxin, which is extremely toxic to some animals even at relatively low concentrations (Lalli and Parsons, 1993). Although the exact cause was uncertain, Cardwell, Olsen, Carr, and Sanborn (1979) reported Pacific oyster (*Crassostrea gigas*) larval and adult die offs in several inlets of Puget Sound in association with the presence of high concentrations of this

dinoflagellates species. The authors also observed a significant reduction in survival of oyster embryos in the presence of *G. splendens* (at concentrations of < 200 cells/ml) in controlled laboratory studies. The authors indicated that although survival could be reduced, there was no correlation between dinoflagellate presence and abnormal development. This finding is consistent with the results of this study, as the proportion normal endpoint showed no effects for either of the affected samples, while survival was clearly impacted (Figure 5, Table 3).

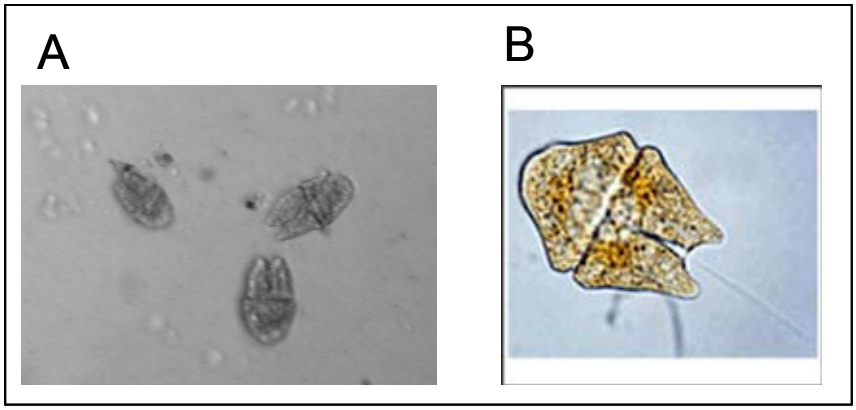

Figure 4. (A) Photograph of dinoflagellates (*Gymnodinium splendens*) observed in samples from 9/27/2005 (Summer/Fall) sampling event in Sinclair and Dyes Inlets. (B) Photograph *of G. splendens* from internet (with permission). Actual cell sizes are 40-80 µm.

Blooms associated with *G. splendens* may also impact dissolved oxygen (D.O.) levels in these inlets, which are highly susceptible to eutrophication (Ecology, 1995). The D.O. levels in our laboratory tests, however, were above critical thresholds in both samples (Appendix C). Therefore, it is unlikely that D.O. concentration impacted survival in the controls.

It is possible, however, that the loading density in the test vials affected embryo survival. Concentrations of dinoflagellates for the M4 and M6 samples were ~1,100 and ~120 cells/ml (~1.1 x 10^6 and ~1.2 x 10^5 cells/L), respectively. To preserve the sample integrity, the samples were not filtered. However, mussel embryos are typically loaded at a density ranging from 15 to 30 embryos/ml to prevent abnormal development (ASTM, 1999, U.S. EPA, 1995a; FAO, 2004), and can develop abnormally at concentrations in excess of 200 embryos/ml (Rosen et al., 2008). Therefore, it's possible that competition for space and/or toxicity associated with toxins secreted by *G. splendens* impacted the number of surviving mussel embryos. The reduced survival in M4, however, still meets survival acceptability of >50% according to the EPA test method (U.S. EPA, 1995a; Appendix B). Furthermore, the high proportion normal (94.2%) for the sample, and the normal dose response associated with the copper additions suggest the test was acceptable.

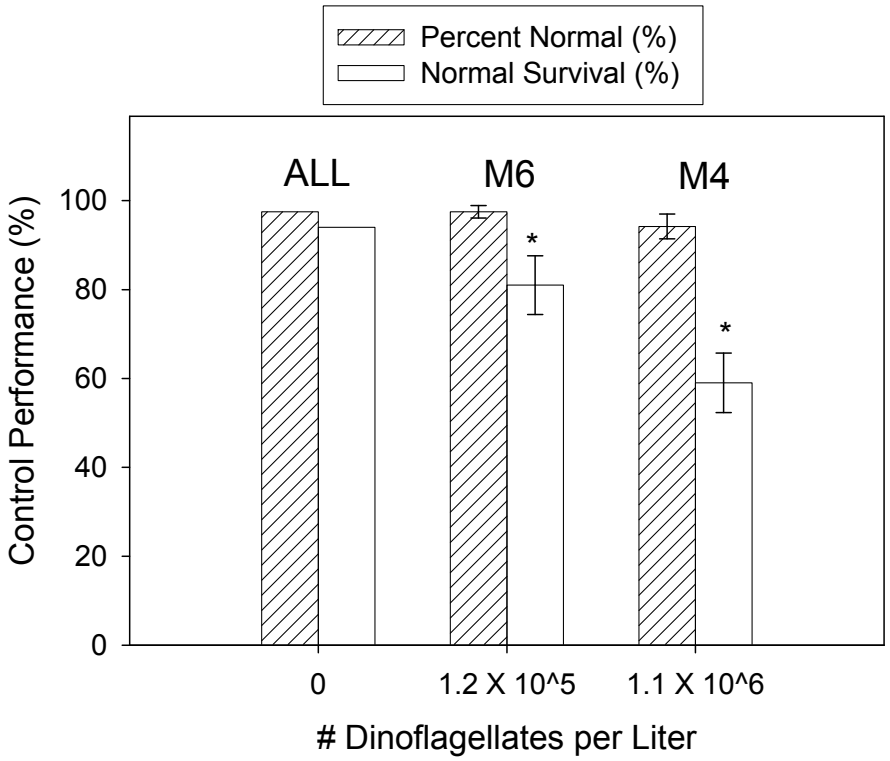

Figure 5. Larval development success of *M. galloprovincialis* embryos in controls (ambient seawater) from samples M6 and M4 from the 9/27/2005 (Summer/Fall) sampling event, in which elevated concentrations of the dinoflagellate *Gymnodinium splendens* were observed, compared with mean control performance for all other ambient site water samples in which no elevated dinoflagellate concentration was observed.

Despite the reduction in control normal survival associated with the plankton bloom in two samples, ambient toxicity at the site was not observed. The absence of ambient toxicity is important because (1) it suggests that ambient conditions of the water body are not toxic to developing mussel embryos, and (2) if ambient toxicity had been observed, it may have confounded the data interpretation from the spiked copper treatments, possibly preventing a WER estimate.

AMBIENT COPPER CONCENTRATION

Copper measurements and other water quality characteristics of unmodified (ambient) site water and laboratory water are summarized in Table 4. Dissolved copper concentrations, measured using STGFAA, in ambient samples were below the national marine chronic AWQC (3.1 µg/L), averaging 1.06 ±0.55 µg/L (±1 s.d.). Dissolved measurements averaged 65% of the total recoverable concentrations, which averaged 1.63 ±0.75 µg/L. These concentrations are consistent with previous monitoring efforts in Sinclair Inlet, where dissolved copper averaged 0.77 µg/L over 19 surveys and three time periods (Katz et al., 2004).

In this study, all 13 ambient samples were measured at SSC Pacific, while a contract laboratory (Battelle Marine Science Laboratory, Sequim, WA) measured duplicates of some of these samples (n = 7). The SSC Pacific samples were measured using STGFAA (via direct injection or preconcentration), while the duplicate samples were measured using Inductively Coupled Plasma

Mass Spectrometry (ICP-MS). A comparison of data for which both types of analyses were conducted indicated that ambient samples measured using STGFAA via direct injection tended to be higher than those measured using ICP-MS, with total recoverable and dissolved copper averaging 41 and 18% higher, respectively (see Table 4).

Table 4. Water quality characteristics for laboratory water (LW) and site waters from Sinclair and Dyes Inlets, Washington. TSS = total suspended solids, DOC = dissolved organic carbon, Tot Cu = total recoverable copper, Diss Cu = dissolved Cu, and ND = values below detection limits. Dashed lines indicate sample was either not collected or not measured.

				SSC-SD[1]		Battelle[2]	
Sample Date	Sample ID	TSS (mg/L)	DOC (mg/L)	Total Cu (µg/L)	Diss Cu (µg/L)	Total Cu (µg/L)	Diss Cu (µg/L)
3/31/2004	LW	ND	0.90	1.0	0.6	-	-
	M3.1	7	1.51	1.2	0.7	1.5	1.1
	M3.3	6	0.92	1.4	1.0	-	-
	P3	6	0.85	2.8	1.5	2.2	1.6
2/9/2005	LW	ND	1.7	2.6	1.8	-	-
	SN12	ND	1.00	2.7	1.6	1.7	1.4
	M3.1	ND	0.90	1.7	1.0	0.9	0.7
	M4	ND	1.10	1.4	1.2	1.0	0.7
	P3	ND	1.10	2.3	1.8	1.6	1.3
	M6	ND	0.80	1.2	0.7	0.7	0.5
9/27/2005	LW	ND	0.90	1.4	1.4	-	-
	SN12	6	1.70	0.9	0.7	-	-
	M3.1	3	1.40	0.9	0.6	-	-
	M4	35	3.60	1.0	0.2	-	-
	P3	2	1.50	2.8	2.1	-	-
	M6	8	2.20	0.9	0.7	-	-

[1]Values were derived by STGFAA (by direct injection) for 3/31/2004 and 2/9/2005 events, and STGFAA (preconcentrated) for the 9/27/05 event
[2]All values derived using ICP-MS.

Linear regression analysis indicated a significant relationship ($p = 0.037$, $r^2 = 0.62$) between the two measurement types for total recoverable metal, while the relationship was not significant ($p = 0.086$, $r^2 = 0.48$) for dissolved metal. However, t-tests indicated a significant difference between datasets for total recoverable metal ($p = 0.036$), while no significant difference was observed among the datasets for dissolved metal ($p = 0.102$).

Additional ambient copper measurements that supported the copper complexation capacity measurements brought the total number of samples to 32 (compared to 13 for those associated with the toxicity tests). The additional measurements were similarly lower than AWQC, with total recoverable and dissolved concentrations averaging 1.3 ±0.5 and 1.0 ±0.4 µg/L, respectively (see Table 8).

TOXICITY FROM COPPER ADDITIONS

When copper was added to lab and site waters, a dose response was observed for all samples. Based on the degree of the dose response, however, it appeared that some samples were more protective than others (e.g., the lab water) against copper toxicity (Figure 6). Toxicity metrics, including EC50 and NOEC/LOEC values, for all toxicity samples, are summarized in Table 5. Raw toxicity results for all test concentrations and samples are tabulated in Appendix D. Nominal toxicity metrics represent the calculated concentrations based on dilution of the stock solution, while total recoverable and dissolved values are based on measured copper results. Dissolved EC50 values for the lab water, which also served as the reference toxicant in most cases, ranged from 6.9 to 8.1 µg/L (geomean = 7.6 µg/L) (Table 5). An additional reference toxicant test (RT) was also conducted with undiluted (salinity = 34 ‰) laboratory water for the 3/31/2004 event, and had a similar result (dissolved EC50 = 8.9 µg/L; Table 5). These values are very similar to lab water results reported in other WER studies using *Mytilus* (e.g., City of San Jose, 1998), suggesting that SIO lab water was representative of other laboratory waters (e.g., Granite Canyon) used in both site-specific (City of San Jose, 1998) and national WQC development (U.S. EPA, 1995b). For example, geometric mean EC50 values for lab waters from two separate WER studies for South San Francisco Bay were 6.3 and 6.9 µg/L, for 1997 and 1991 studies, respectively (City of San Jose, 1998).

The total recoverable and dissolved EC50 values obtained from the site water from Sinclair and Dyes Inlets were always higher than those obtained for the lab waters. This finding resulted in WER values greater than 1, indicating that the water in Sinclair and Dyes Inlets buffer against copper toxicity to a greater degree than laboratory water when EC50s are expressed as total recoverable or dissolved copper. On average, EC50s from site water were 59 and 41% higher than laboratory water, based on total recoverable and dissolved metal measurements, respectively. Dissolved EC50s were generally, but not always lower than total recoverable EC50s, averaging 74.1% of the total. Across surveys, there was no consistent trend in the relative magnitudes of EC50s among the test sites. That is, no one site always stood out as having the most or least amount of protection from copper, based on EC50 values. However, EC50s were correlated with both TSS and DOC.

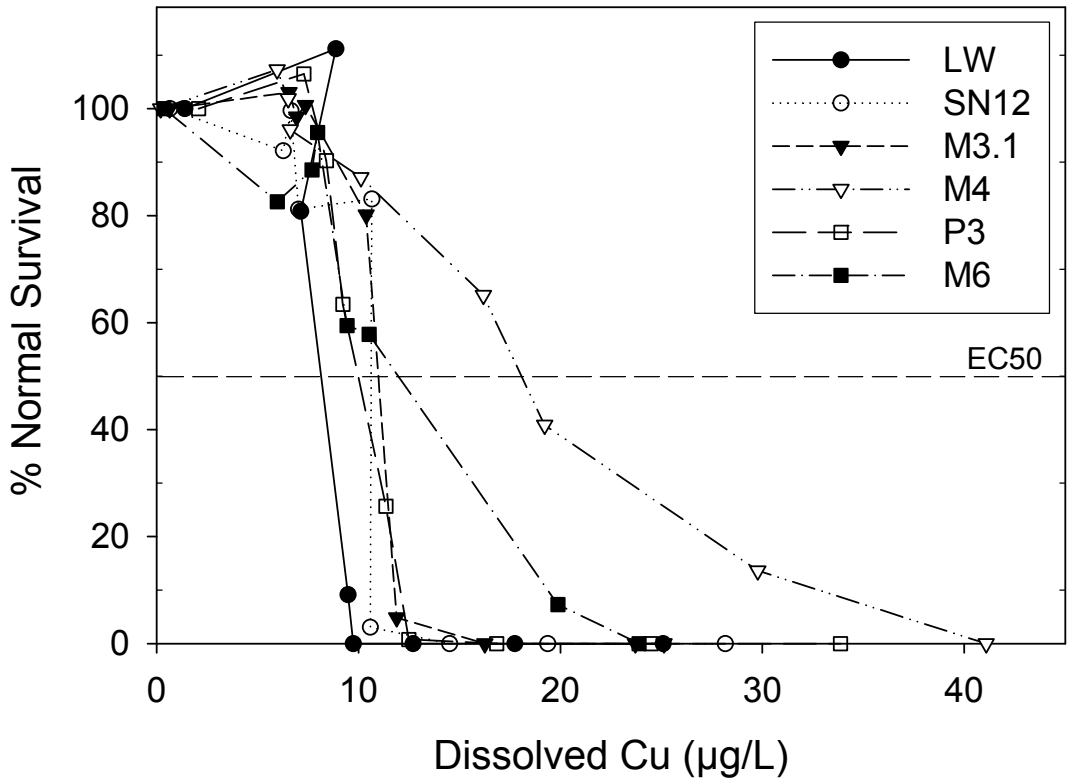

Figure 6. Dose response curves generated from copper additions to lab water (LW) and site water samples from Sinclair and Dyes Inlets for the 9/27/2005 (Summer/Fall) sampling event, shown as an example of the variation in the level of protection against copper toxicity that the different water samples possessed.

Table 5. Summary of no observable effect concentrations (NOEC), lowest observable effect concentrations (NOEC), median effect concentrations (EC50) and the 95% confidence interval (C.I.) about the EC50 from M. galloprovincialis embryogenesis toxicity tests conducted with copper additions to laboratory water (LW), reference toxicant tests (RT), and site waters from Sinclair and Dyes Inlets, Washington. Data are expressed as nominal and measured (total recoverable and dissolved) copper.

Sampling $		ample	Nominal (µg/L)				Total Recoverable (µg/L)				Dissolved (µg/L)			
Date	Location	ID	NOEC	LOEC	EC50	95 % C.I.	NOEC	LOEC	EC50	95 % C.I.	NOEC	LOEC	EC50	95 % C.I.
3/31/2004	SIO	LW 8.	3	11.8	10.1	9.2-10.7	9.4	12	10.7	10.3-11.1	6.9	9.4	8.1	7.8-8.5
	SIO	RT	8.3	11.8	11.1	11.0-11.3	9.7	11.4	11.6	11.5-11.8	6.7	9.6	8.9	8.8-9.0
	Sinclair Inlet	M3.1	11.8	17	15.9	6.9-19.9	11.2	17.2	15.5	15.3-15.8	9.2	13.3	12.3	9.1-13.9
	Sinclair Inlet	M3.3	11.8	17	16.4	16.1-16.6	12.7	19.5	18	17.8-18.3	9.2	13.3	12.6	12.4-12.7
	Sinclair Inlet	P3	8.3	11.8	12.9	11.9-13.9	10.1	12.6	14.3	13.3-15.3	5.6	9.2	10.3	10.1-10.5
2/9/2005	SIO	LW/RT	4.1	5.9	6.1	5.8-6.3	6.6	10.0	9.8	9.7-9.9	5.4	7.2	6.1	3.0-7.3
	Sinclair Inlet	SN12	5.9	8.4	8.3	6.0-9.7	12.1	13.3	13.4	12.1-15.8	7.2	8.4	8.5	7.3-10.4
	Sinclair Inlet	M3.1	5.9	8.4	10.2	9.3-11.0	8.4	12.6	13.9	13.8-14.0	6.1	8.1	9.8	9.2-10.4
	Sinclair Inlet	M4	5.9	8.4	9.7	9.6-9.8	8.7	12.0	13.2	13.1-13.2	6.2	8.3	9.5	9.4-9.6
	Sinclair Inlet	P3	5.9	8.4	8.0	7.5-8.4	9.3	13.4	12.9	11.8-13.5	6.9	9.4	9.0	8.9-9.1
	Dyes Inlet	M6	5.9	8.4	10.0	9.3-10.7	9.6	11.6	13.9	12.8-14.9	5.3	7.7	8.3	8.2-8.4
9/27/2005	SIO	LW/RT	2.9	4.1	4.7	4.4-4.9	<7	7.0	7.5	7.5-7.6	<7.1	7.1	7.9	7.1-8.4
	Sinclair Inlet	SN12	4.1	5.9	9.7	8.0-11.0	7.5	8.8	13.7	12.7-14.4	6.7	7.0	9.9	9.8-10.0
	Sinclair Inlet	M3.1	5.9	8.4	9.5	9.3-9.6	8.1	11.0	12.3	12.1-12.4	7.4	10.4	10.7	10.6-10.8
	Sinclair Inlet	M4	8.4	17.2	21.2	17.8-23.9	12.4	20.8	25.3	22.7-27.5	10.1	16.2	18.3	16.3-20.0
	Sinclair Inlet	P3	4.1	5.9	6.5	5.7-7.2	10.4	13.7	11.7	10.6-12.8	9.2	11.4	9.9	9.0-10.6
	Dyes Inlet	M6	5.9	8.4	19.9	6.6-23.2	9.5	11.6	16.8	8.3-23.4	8.0	9.4	12.5	8.5-15.7

COPPER MEASUREMENTS IN TOXICITY TESTS

Individual copper measurements for each of the toxicity tests are shown in Appendix E. In general, total recoverable concentrations were higher than nominal (targeted) concentrations at the low concentrations, while they were close to nominal for the higher concentrations. While total recoverable concentrations overall averaged 35% higher than nominal, they averaged only 16% over the target at the highest concentration tested. The relatively large discrepancy for low concentrations is very likely due to the presence of copper in the ambient sample that was not be accounted for in the targeted concentration. Total recoverable concentrations were nearly always higher than dissolved concentrations. This was expected because of binding to suspended particles, which are removed for dissolved measurements. The ratio between dissolved and total recoverable metal was similar to the ambient samples, averaging 77 ±14% overall, and 70 ±8%, 76 ±18%, and 82 ±11% for the 3/31/2004, 2/9/2005, and 9/27/2005 events, respectively. No clear trend among the dissolved: total fraction was observed among the different test concentrations.

WATER-EFFECT RATIOS AND SITE-SPECIFIC CRITERIA

The water-effect ratio (WER) is calculated simply by dividing the site water EC50 by the associated lab water EC50. WERs for each site water sample are presented in Table 6 by sampling event. Total recoverable WERs, which were relatively close to nominal WERs, ranged from 1.32 to 3.37. Dissolved WERs ranged from 1.20 to 2.32 (Table 6, Figure 7). As with the EC50s, no trend in the WER magnitude was apparent among sample locations or across surveys. Although WERs associated with M4 and M6 were relatively high for the 9/27/2005 sampling event (Table 6), no statistical differences were observed when comparing WERs within a sampling event ($p= 0.683$ [tot recov], $p = 0.254$ [dissolved]) or among sampling events ($p = 0.430$ [dissolved], $p = 0.074$ [tot recov]) using one-way ANOVA ($\alpha = 0.05$).

In addition, no statistical difference was observed between WERs from Sinclair Inlet compared to WERs from Dyes Inlet ($p = 0.720$ [tot recov], $p = 0.821$ [dissolved]) using t-tests ($\alpha = 0.05$). Furthermore, the largest differences among total recoverable and dissolved WERs spanned a factor of only 2.6 and 1.9, respectively. Since all WERs were within a factor of 3, all sampling locations and sampling events could be used in the final WER calculation for classification as one site (U.S. EPA, 1994a). The final total recoverable and dissolved WERs, calculated as the geometric mean of all 13 WERs derived, were 1.63 and 1.41, respectively (Table 6). The WERs were then multiplied by the national AWQC to provide estimates for site-specific WQC for copper in Sinclair and Dyes Inlets (Table 7). The WER-derived dissolved site-specific WQC were 88.6% of the total recoverable site-specific WQC. If the site-specific WQC are to be applied directly to a permit, the total recoverable WER may be considered most applicable. If the WER is to be used to adjust the national AWQC for Sinclair and Dyes Inlets, the dissolved values are more appropriate, as AWQC are currently expressed in terms of dissolved concentrations.

Table 6. Nominal, total recoverable, and dissolved water effect ratios (WER) for copper for sites in Sinclair and Dyes Inlets, Washington, using *Mytilus galloprovincialis* embryo-larval development toxicity tests.

		Water Effect Ratio		
Sampling Date	**Sample ID**	**Nominal**	**Total Recov.**	**Dissolved**
3/31/2004	M3.1	1.57	1.45	1.52
	M3.3	1.62	1.68	1.56
	P3	1.28	1.34	1.27
	Geo Mean	1.48	1.48	1.44
2/9/2005	SN12	1.36	1.37	1.23
	M3.1	1.67	1.42	1.43
	M4	1.59	1.34	1.38
	P3	1.31	1.32	1.30
	M6	1.65	1.42	1.20
	Geo Mean	1.51	1.37	1.31
9/27/2005	SN12	2.07	1.82	1.26
	M3.1	2.02	1.63	1.36
	M4	4.53	3.37	2.32
	P3	1.39	1.56	1.26
	M6	4.25	2.24	1.58
	Geo Mean	2.57	2.03	1.51
Final WER	**Geo Mean**	**1.84**	**1.63**	**1.41**

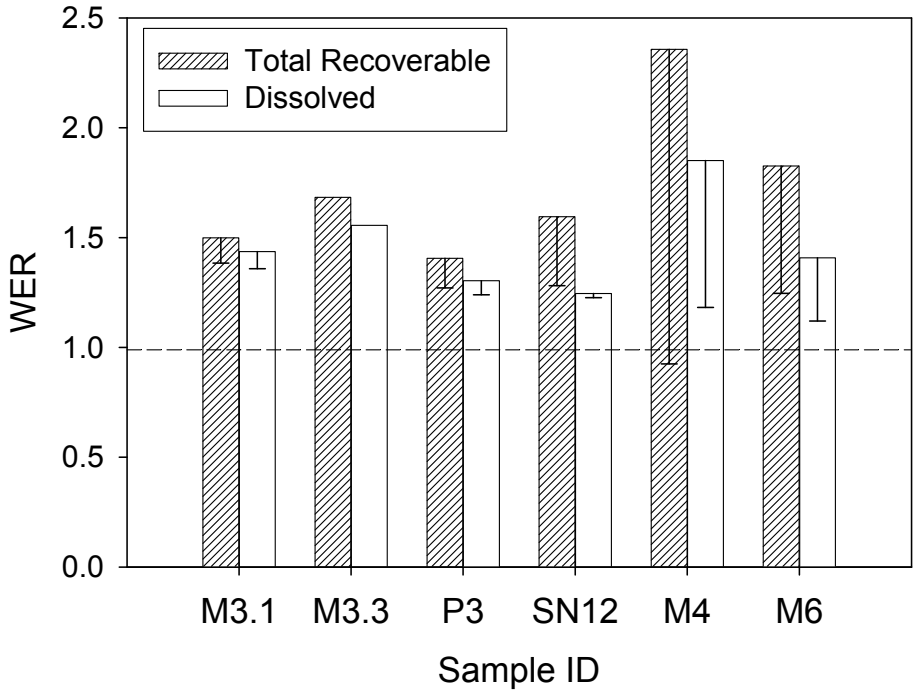

Figure 7. Mean (±1 s.d.) total recoverable and dissolved water effect ratios (WERs) at each of the sites in Sinclair and Dyes Inlets, from a total of three sampling events. Values above 1 (as indicated by the dashed line) suggest protection at the site is greater than that provided by laboratory water, such as that used in AWQC development.

Table 7. National ambient water quality criteria (AWQC) and site-specific WQC for Sinclair and Dyes Inlets, as calculated from this study using the WER procedure (U.S. EPA, 1994a).

	National AWQC	WER-Derived Site-Specific WQC	
	Dissolved	Total Recoverable	Dissolved
Acute (µg/L)	4.8	7.80	6.77
Chronic (µg/L)	3.1	5.04	4.37

TOXICITY AND TSS

The bioavailability and potential for toxicity of copper is dependent on various water quality characteristics, including TSS and DOC. In this study, TSS concentrations averaged 9.1 ±10.6 mg/L in site water, but were always non-detectable in the filtered lab waters (Table 4). Overall, suspended solids significantly correlated with both total recoverable ($p < 0.001$; $r^2 = 0.81$) and dissolved ($p < 0.001$; $r^2 = 0.87$) EC50s. The relationship was particularly strong for the 9/27/2005 event ($p = 0.004$, $r^2 = 0.90$ [total recov]; $p = 0.003$, $r^2 = 0.93$ [dissolved]), in which a relatively broad range in TSS concentrations (2 to 35 mg/L) was measured (Table 4). Suspended solids data were not available for the 2/9/2005 event, as insufficient sample volume resulted in high method detection limits (5 mg/L). No correlation between TSS and EC50 was observed for the 3/31/2004 event. This is likely due to too few data points (only three site water samples) and only modest differences among TSS concentrations as well as EC50 values for that event. Correlation between TSS and total recoverable EC50s are expected, as the presence of particulates provides binding sites that can potentially decrease copper bioavailability, and therefore, observed toxicity to organisms (Erickson et al., 1996).

TOXICITY AND DOC

As with TSS, dissolved EC50s from this study were significantly correlated ($p < 0.001$, $r^2 = 0.74$) to DOC concentrations (Figure 8), which averaged 1.43 ±0.77 (range 0.80 to 3.6) mg/L. The lab water DOC value from the 2/9/2005 event was not included in the regression analysis because it was uncharacteristically high for SIO laboratory water, and did not correspond with observed toxicity. Therefore, it was believed to be an outlier. However, without deletion of that value, the relationship was still significant ($p < 0.001$, $r^2 = 0.55$). The DOC concentrations observed for this study are similar to those for several other estuaries for which dissolved WERs of less than 2 were achieved (e.g., U.S. EPA, 1994c; CH2M Hill, 2000; Rosen, Rivera-Duarte, Kear-Padilla, and Chadwick, 2005), while generally high total organic carbon (DOC was not measured) concentrations (range < 2.5 to > 9 mg/L) coincided with a relatively high dissolved WER of 2.77 for South San Francisco Bay (City of San Jose, 1998).

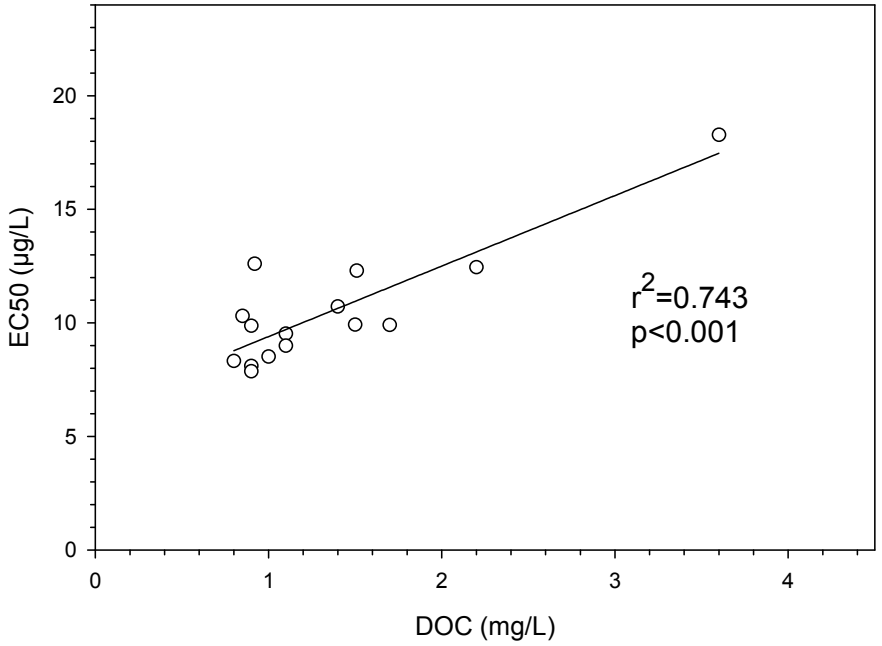

Figure 8. Relationship between median effect concentration (EC50) and dissolved organic carbon (DOC) concentration from Sinclair and Dyes Inlets samples (n = 15).

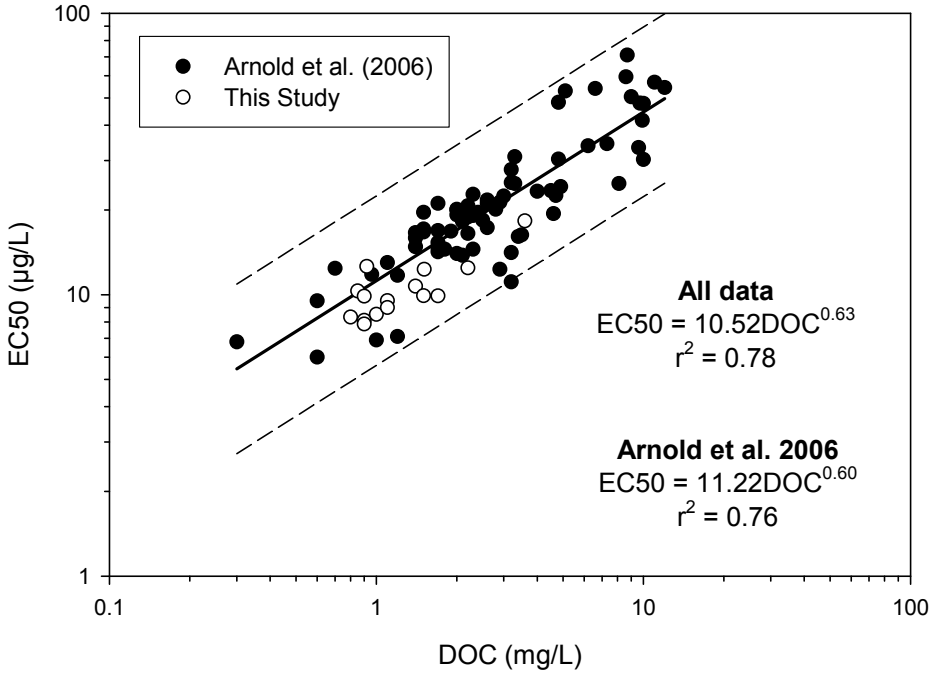

Figure 9. Plot of pooled results from this study and that of Arnold, Cotsifas, and Corneillie (2006), depicting the relationship between DOC concentration and EC50 value for *Mytilus galloprovincialis* embryo-larval development. Solid line is the line of best fit, and dashed lines represent line of best fit, plus or minus a factor of 2. Regression equations shown are based on Arnold, Cotsifas, and Corneillie (2006), and combined with data from Sinclair and Dyes Inlets (all data).

The dependence of metal toxicity on DOC concentration in aquatic environments is well studied (Knezovich, Harrison, and Tucker, 1981; Meador, 1991; Kim, Ma, Allen and Cha, 1999; Arnold, Cotsifas, and Corneillie, 2006). Recently, regression equations to predict copper EC50s based on DOC concentration have been determined from results of several WER studies that employed *M. galloprovincialis* embryo-larval development as the test endpoint (Arnold, 2005, Arnold, Cotsifas, and Corneillie, 2006). The use of these equations to predict saltwater site-specific WQC for copper are currently being considered for implementation by the U.S. EPA until a saltwater Biotic Ligand Model (BLM) is available. The relationship between EC50 and DOC from Arnold, Cotsifas, and Corneillie (2006) was described as

$$EC50 = 11.22DOC^{0.60} \ (p<0.001, r^2=0.76, n=75 \quad (1)$$

When the Sinclair and Dyes Inlet samples (n=15) are plotted with these data, the relationship remains equally significant, and becomes:

$$EC50=10.52DOC^{0.63} \ (p<0.001, r^2=0.78, n=90) \text{ (Figure 9).} \quad (2)$$

It is interesting to note that in this study, however, empirically derived EC50 values were generally 25% lower than they would have been predicted using the equation determined by Arnold et al. (2006). This could be due to the fact that regression equations developed by Arnold et al. (2006) covered a relatively broad range in DOC concentrations (<1 to ~12 mg/L), while the sites in Sinclair and Dyes Inlets were characterized by relatively low DOC (<1 to 3.6 mg/L). It should be noted, however, that the model aims to predict the EC50 within a factor of 2, which was easily achieved for all samples in this study (Figure 9).

Because the genus mean acute value (GMAV) for *Mytilus* is equivalent to the final acute value (FAV) used for derivation of national WQC for copper in saltwater (U.S. EPA, 1995b), division of equation 1 by the product of the acute-to-chronic ratio (ACR) of 3.127 and the national chronic WQC (3.1 µg/L;U.S. EPA, 1995b) results in an equation that can directly predict the WER from the geometric mean of the DOC concentrations measured at a site:

$$WER_{DOC} = 1.16DOC^{0.60} \quad (3)$$

Using equation 3, measured and DOC-predicted WERs differed by less than 5% (Figure 10). This close relationship provides an additional line of evidence that a site-specific criterion for copper is justified for Sinclair and Dyes Inlets, and also successfully demonstrates the utility of the DOC model as a means of predicting WERs using simple and less costly means compared to toxicity testing and the associated chemical analyses involved in formal WER studies.

The availability of additional DOC data from other sampling events conducted in 2005 allowed for the derivation of DOC-predicted WERs and site-specific WQC with a more comprehensive data set (Brandenberger et al., 2006). Combined with our data, a total of 117 data points were available from 7 sampling events for 26 sampling stations in Sinclair and Dyes Inlets. DOC concentration averaged 1.18 ±0.33 mg/L (geomean = 1.14 mg/L), about 20% lower than the average calculated from samples used in the WER study. The lower DOC concentrations associated with the larger dataset, therefore, yielded a lower final DOC-derived WER of 1.27 (geometric mean)(Figure 10). Although more conservative, this calculated WER was not statistically different (t-test, $p > 0.05$) from the WER derived using toxicity testing.

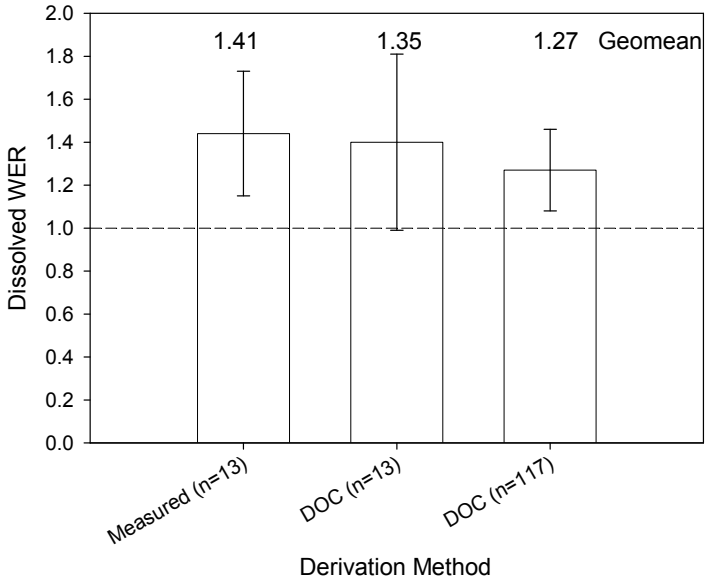

Figure 10. Measured dissolved WERs compared with predicted dissolved WERs using DOC-regression model for *Mytilus galloprovincialis*. Bars show arithmetic mean and standard deviation.

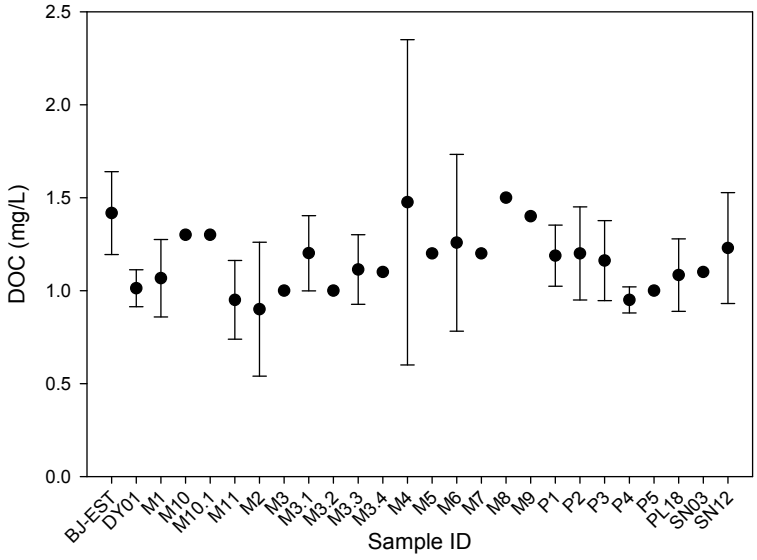

Figure 11. Mean ±SD dissolved organic carbon (DOC) measurements (n = 117) for 26 marine sampling stations in Sinclair and Dyes Inlets during 2005 ENVVEST sampling events.

TOXICITY AND COMPLEXATION CAPACITY

Free Copper Ion

The lack of toxicity observed in the ambient site water samples tested in this study was associated with free copper ion concentrations ($Cu(II)_{aq}$) over an order of magnitude lower than those expected to result in toxicity. As explained below, mussel EC50 values coincided with an average (± 1s.d) pCu ($-(\log [Cu(II)_{aq}])$) of 11.0 ± 0.4 (range 10.7 to 11.8; Table 8), which is referred to as the pCu_{tox}, and defines the free copper ion concentration that results in the observed toxic effect. In contrast, pCu values measured in ambient samples at the beginning of the CuCC titrations (e.g., prior to any copper addition) had an average value of 12.6 ±0.6 (range 11.2 to 14.1), which is over an order of magnitude lower than the pCu_{tox}. The free copper ion concentrations measured in Sinclair and Dyes Inlets are similar to those from other estuaries (see Blake, Chadwick, Zirino, and Rivera-Duarte, 2004). Interestingly, some of the highest pCu values (i.e., lowest free copper ion concentrations) were associated with the M4 and M6 samples from the 9/27/2005 event (Table 8). This could be explained by the fact that those samples also possessed the highest TSS and DOC concentrations, providing more binding sites for the free Cu ions. These data provide yet another line of evidence that reduced normal survival in the controls from M4 and M6 for that event was unlikely associated with copper exposure.

There are three measurements considered suspect; these correspond to samples SN12, M4 and P3 of 2/9/2005. These samples had the three lowest measured values for the initial pCu (i.e., highest free copper ion concentrations), and the CuCC titrations did not have the expected inflection point indicative of the CuCC value; therefore, the information from these samples is not included in the study.

The concentration of free copper ion associated to the EC50 (pCu_{tox}) was calculated using the CuCC titrations. In general, the toxicity testing and CuCC measurement consist of similar copper additions to the sample water, with the difference in the actual measurement done. In toxicity testing the percentage of organisms that had reached the expected development is measured. In a CuCC titration the change in the pCu is measured. These measurements are shown for sample SN12 of 9/27/2005 in Figure 12.

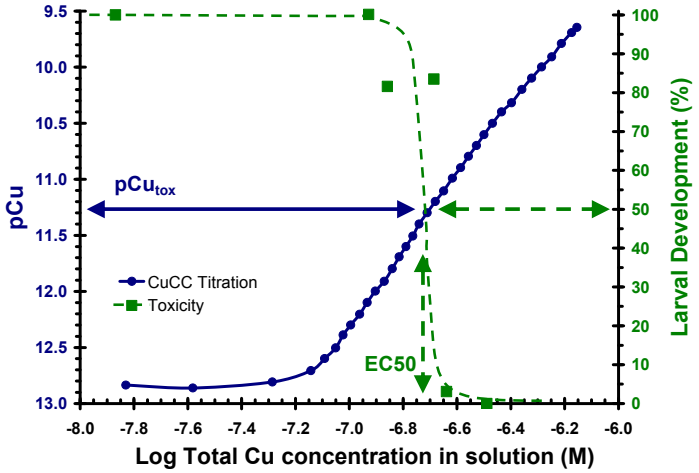

Figure 12. Change in larval development and pCu measured in the toxicity testing and CuCC titration for sample SN12 of 9/27/2005.

Table 8. Copper complexation capacity and free copper ion concentration data and predicted values.

Sampling date	Station ID	Total [Cu] (µg/L)	Dissolved [Cu] (µg/L)	CuCC Titration Initial pCu	CuCC [Cu] (µg/L)	CuCC [Cu] (nM)	Predictions with pCutox and correlation between between Diss vs Tot EC50				Predictions from correlation beween CuCC vs DissEC50	
							pCu_{tox} Total	Total Di ssolved EC50 (µg/L)	Dissolved EC50 (µg/L)	Dissolved WER	Dissolved EC50 (µg/L)	Dissolved WER
31-Mar-04	**M3.1**	1.5	1.0	12.1	11.76	185	10.8	14.22	10.4	1.28	11.96	1.48
	M3.3			12.0	13.73	216	10.7	15.14	11.0	1.36	13.11	1.62
	P3	2.2	1.6	12.1	8.50	134	10.9	12.68	9.3	1.15	10.04	1.24
9-Feb-05	**SN12**	1.7	1.4	11.2								
	M3.1	0.9	0.7	12.4	5.07	80	11.0	13.35	9.8	1.60	8.02	1.32
	M4	1.0	0.7	11.4								
	P3	1.6	1.3	11.5								
	M6	0.7	0.5	12.7	6.19	97	10.8	12.21	9.0	1.47	8.68	1.42
2-Mar-05	**SN12**	1.3	1.0	12.4	5.30	83		10.32	7.7	1.10	8.16	1.17
	M3.1	0.7	0.6	12.3	4.35	68		10.79	8.0	1.14	7.59	1.08
	M4	1.2	1.0	12.7	6.69	105		11.68	8.6	1.23	8.97	1.28
	P3	2.5	2.2	12.5	5.97	94		12.76	9.4	1.34	8.55	1.22
	M6	0.7	0.6	12.7	5.26	83		11.05	8.2	1.17	8.13	1.16
20-Jun-05	**SN12**	1.3	1.5	13.9	8.16	128		14.76	10.7	1.53	9.84	1.41
	M3.1	1.0	1.0	12.8	8.44	133		15.07	10.9	1.56	10.00	1.43
	M4	1.0	0.9	12.8	8.23	130		16.00	11.6	1.66	9.88	1.41
	P3	1.4	0.6	12.5	7.13	112		14.26	10.4	1.48	9.23	1.32
	M6	0.9	0.7	12.1	7.80	123		11.45	8.5	1.21	9.63	1.38
	BJEST	1.4	1.0	12.5	7.73	122		15.30	11.1	1.59	9.59	1.37
	DY01	0.8	0.7	12.2	10.49	165		12.99	9.5	1.36	11.21	1.60
	M3.2	1.0	1.0	13.2	4.05	64		14.88	10.8	1.55	7.42	1.06
	P1	1.2	1.1	12.4	4.87	77		10.85	8.1	1.15	7.90	1.13
	P2	1.5	1.3	12.8	3.93	62		13.49	9.9	1.41	7.35	1.05
27-Sep-05	**SN12**	0.9	0.7	12.8	6.57	103	11.2	15.15	11.0	1.39	8.91	1.13
	M3.1	0.9	0.6	12.6								
	M4	1.0	0.2	13.3								
	P3	2.8	2.1	12.2	12.68	199	11.8	17.96	12.9	1.64	12.50	1.58
	M6	0.9	0.7	14.1								
	BJEST	1.3	0.8	13.3	7.73	122		20.93	15.0	1.89	9.59	1.21
	DY01	0.7	0.6	12.6	5.13	81		13.52	9.9	1.25	8.06	1.02
	M3.2	2.2	1.2	12.8	8.25	130		16.11	11.7	1.48	9.89	1.25
	P1	1.5	0.7	12.5								
	P2	1.8	1.4	13.3	5.61	88		14.95	10.9	1.38	8.34	1.06
Overall statistics												
Average		1.3	1.0	12.6	7.29	115	11.0	13.92	10.2	1.40	9.33	1.28
Geomean		1.2	0.9	12.6	6.89	109	11.0	13.73	10.0	1.39	9.22	1.27
Standard deviation		0.5	0.4	0.6	2.59	41	0.4	2.39	1.6	0.20	1.52	0.18
Maximum		2.8	2.2	14.1	13.73	216	11.8	20.93	15.0	1.89	13.11	1.62
Minimum		0.7	0.2	11.2	3.93	62	10.7	10.32	7.7	1.10	7.35	1.02
n		32	32	33	26	26	7	26	26	26	26	26

As illustrated in Figure 12, EC50 is calculated as the concentration expected to produce normal development in 50% of the organisms tested. The pCu associated with the EC50 (i.e., pCu_{tox}) is estimated from the CuCC titration curve, by identifying the pCu measured at the EC50 total copper concentration. Following the FIAM, the pCu_{tox} should be in a relatively narrow range, as was estimated here with an average of 11.0 ±0.4. This pCu_{tox} is consistent with previous findings in San Diego Bay, California, where a pCu of ~11 was estimated to result in toxicity to marine invertebrate larvae (Rivera-Duarte et al., 2005).

Copper Complexation Capacity

CuCC measurements are shown in Table 8. As indicated above, the titration of samples SN12, M4 and P3 from 2/9/2005 resulted in curves with no inflection point; therefore, CuCC was not calculated for these samples. In the case of samples M3.1, M4, M6, and P1 from 9/27/2005, the titration curves had an unexplained shape, which for the case of sample P1 was observed in both an unfiltered and a 0.45-µm filtered aliquot (Figure 13). Therefore, it was considered that estimation of CuCC for those samples would be misleading, so they were not used. It was noted that these samples had a strong odor similar to that of seaweed, possibly due to the high concentrations of toxic algae (*G. splendens*) observed in samples collected during the 9/27/2005 event. The measured CuCC values in general cover a wide range (average = 7.29 ±2.59 µg/L, range = 3.93 to 13.73 µg/L), indicating a range in buffering characteristics throughout the study area. Ultimately, in accordance with the FIAM, these values indicate that the studied waters have a range in the capacity to assimilate inputs of copper before reaching the toxic endpoint (EC50). This range in buffering capacity is shown in Figure 14, where stations M4, M6, P1, and P2 show the narrowest temporal ranges, and stations M3.1 and P3 the widest temporal range.

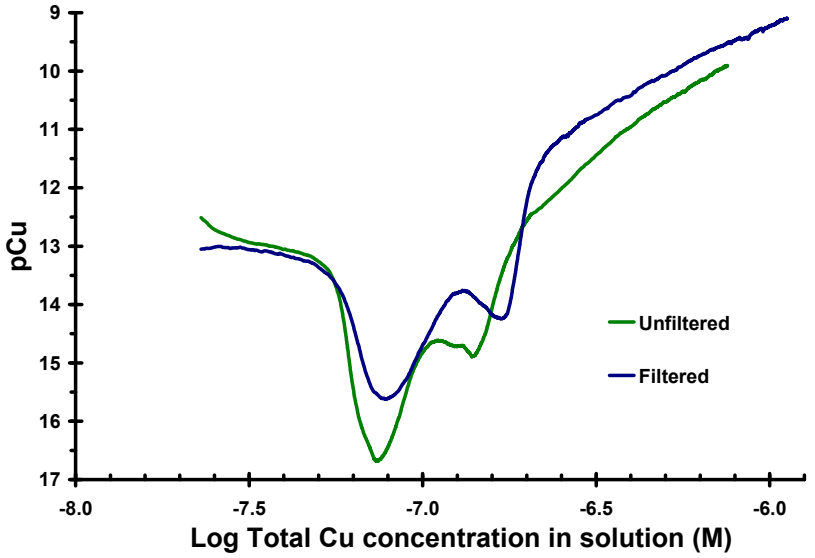

Figure 13. CuCC titrations for sample P1 from 9/27/2005 showing the shape of the resulting curve for the unfiltered and filtered aliquots. Compare to the shape for sample SN12 of 9/27/2005 in Figure 12

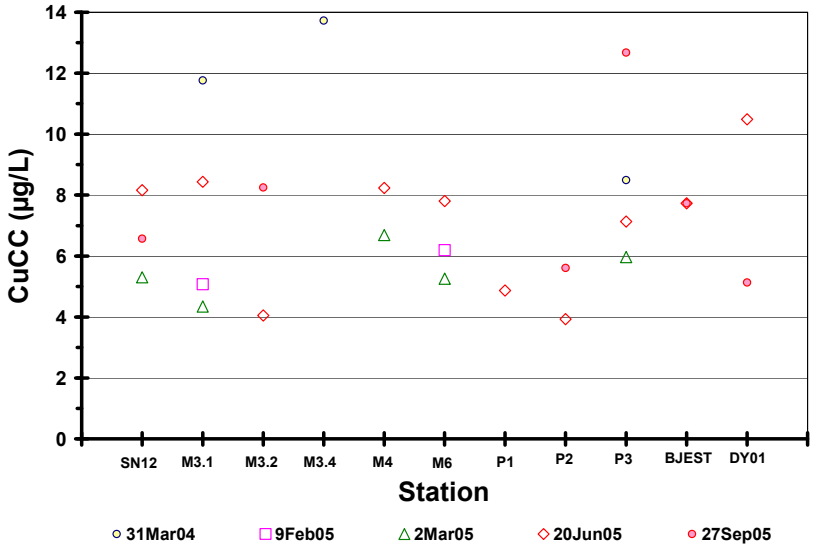

Figure 14. Range in copper complexation capacity (CuCC) measured in this study. CuCC is plotted per station to provide information of both the spatial and temporal range.

Estimation of WERs from CuCC

Measurements of CuCC were done as an analytical procedure to estimate WERs in samples where no toxicity testing was performed. This was accomplished following two different approaches: (1) estimation of total EC50s from pCu_{tox} and application of the relationship between lab-measured dissolved EC50 and lab-measured total EC50, and by (2) correlation between CuCC and the lab-measured dissolved EC50.

(1) Estimation of Dissolved WER from pCu_{tox}

As explained above, pCu_{tox} is the free copper ion concentration that corresponds to the total or dissolved copper concentration at the EC50. Estimation of the pCu_{tox} is achieved by combining a biological measurement (i.e., toxicity test) with a chemical measurement (i.e., CuCC titration). In this case, the biological and chemical measurements from seven different samples were paired to give a pCu_{tox} of 11.0 ± 0.4 with a range from 10.7 to 11.8 (Figure 12). The pCu_{tox} was then used to estimate the total EC50 in all of the samples with acceptable CuCC titrations as shown in Figure 12, where the pCu_{tox} is paired to the total copper concentration of the EC50. This pairing resulted in predicted total EC50s averaging 13.92 ±2.39 µg/L (range = 10.32 to 20.93 µg/L), which differs by only 7% of the average for lab measured total EC50s (average = 15.0 ±3.6 µg/L, range = 11.7 to 25.3 µg/L).

Because dissolved WERs require the determination of the dissolved EC50, the next step was to estimate the dissolved EC50 from the estimated total EC50. This was done using the correlation of $y = 0.686x + 0.608$ ($r^2 = 0.866$) observed between the lab dissolved EC50s and the lab total EC50s (Figure 15). This relationship resulted in an estimated average dissolved EC50 of 10.2 ± 1.6 µg/L (range = 7.7 to 15.0 µg/L), which is once again approximately only 7% different from the average lab measured dissolved EC50 of 10.9 ±2.6 µg/L (range = 8.3 to 18.3 µg/L).

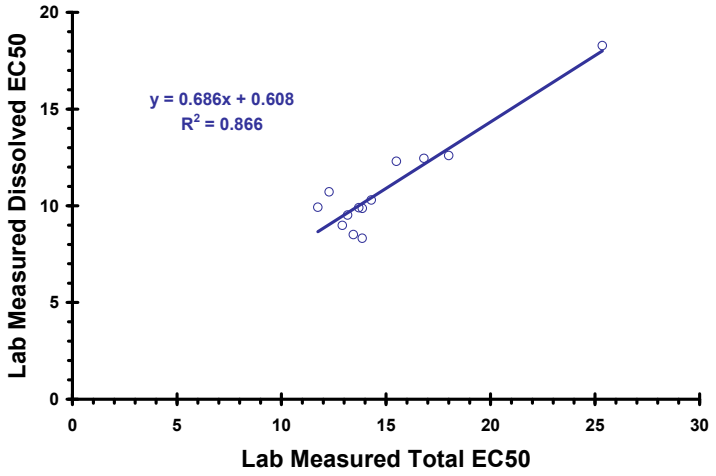

Figure 15. Correlation between dissolved and total EC50 measured in the laboratory.

Determination of the WER involves a comparison between the toxicity measured in the water of interest (site water) and laboratory water. The lab water test results from the WER study for which toxicity testing was done yielded dissolved EC50 values of 8.1 µg/L on 3/31/2004, 6.1 µg/L on 2/9/2005, and 7.9 µg/L on 9/27/2005 (Table 5). These values were used for the samples corresponding to the same sampling dates. Due to the absence of lab water EC50s for 3/2/2005 and 6/20/2005 sampling events, a value of 7.0 µg/L (average of lab water EC50s from 2/9/2005 and 9/27/2005) was used for samples associated with those dates for the purposes of pCu_{tox} WER estimation for those samples. Estimated dissolved WERs using pCu_{tox} averaged 1.41 ±0.17, which was essentially identical to those produced by toxicity testing (average = 1.44 ±0.29, range = 1.2 to 2.3). When an additional 19 data points not associated with the toxicity tests were included, the pCu_{tox} estimated dissolved WER averaged 1.40 ±0.20 (range = 1.10 to 1.89), suggesting that water quality characteristics of water not tested as part of the WER study were similar to the three events for which toxicity testing was conducted (Figure 16).

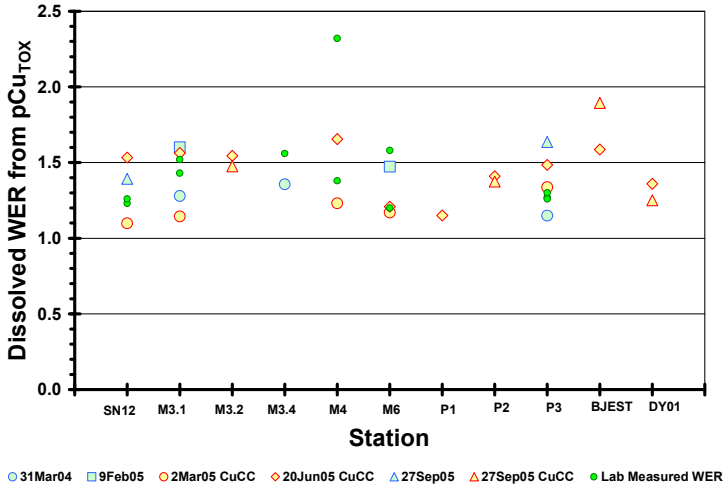

Figure 16. Dissolved WERs estimated following the pCu_{tox} approach. Estimated values agree with the range measured by laboratory experiments, but cover a wider number of stations and sampling events.

(2) Estimation of Dissolved WER from CuCC

A more direct approach to estimate dissolved WERs from CuCC is by correlation with lab measured dissolved EC50s. The agreement between EC50 and CuCC has been observed in San Diego Bay (Rivera-Duarte et al., 2005). In this case, the CuCC measurements for which toxicity tests were also performed were plotted against dissolved EC50 values, resulting in a significant positive relationship ($y = 1.699x - 8.556$; $r^2 = 0.538$; Figure 17). The regression equation generated from this relationship was then used to predict the dissolved EC50 values and WERs for those samples for which no toxicity tests were performed, resulting in a larger dataset. The values used for the SIO lab water are those described in the previous section. The resulting estimated dissolved WERs averaged 1.40 ±0.18 for those samples for which toxicity testing was conducted, yet were slightly lower when all 26 samples (with and without toxicity testing) were included, averaging 1.28 ±0.18 with a range from 1.02 to 1.62 (Figure 18). Interestingly, this was also the case when DOC samples beyond those used in toxicity testing were used to predict WERs using the DOC-toxicity model (Figure 10).

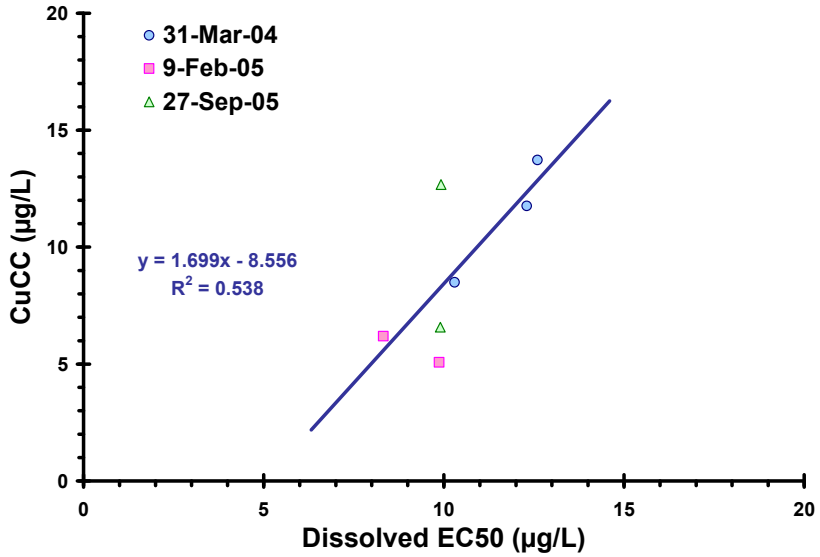

Figure 17. Relationship between copper complexation capacity (CuCC) and dissolved median effect concentration (EC50) from *Mytilus galloprovincialis* embryo-larval toxicity tests with copper.

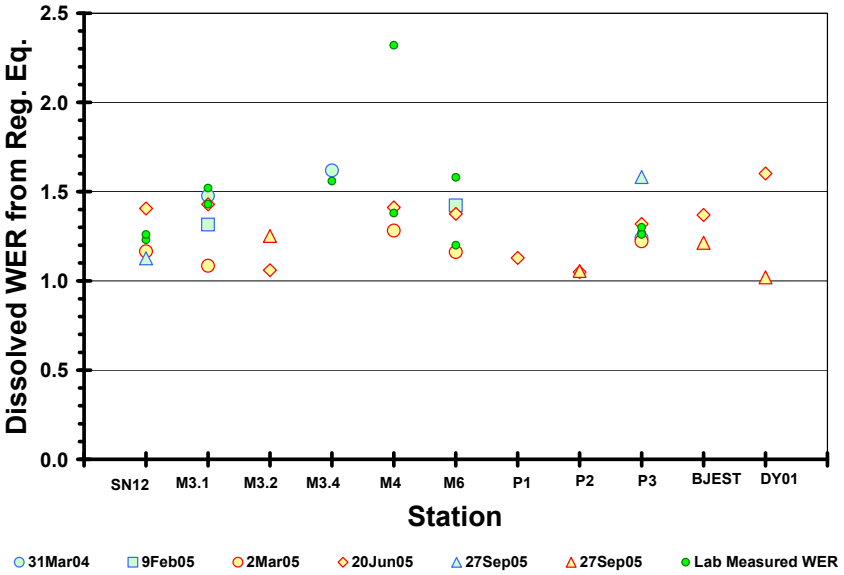

Figure 18. Dissolved WERs estimated following the CuCC approach. Estimated values overlap with the lower range measured by laboratory experiments, but covers a wider number of stations and sampling events.

Advantages and Disadvantages of the pCu_{tox} and CuCC approaches

The two approaches provided dissolved WERs in good agreement to those obtained following the U.S. EPA approved procedure with laboratory toxicity testing (Figure 19). Although not statistically significant ($p > 0.05$), some minor differences among the approaches became apparent when additional data (e.g., samples for which not toxicity testing was conducted) were generated with the intent of providing a more comprehensive WER estimate.

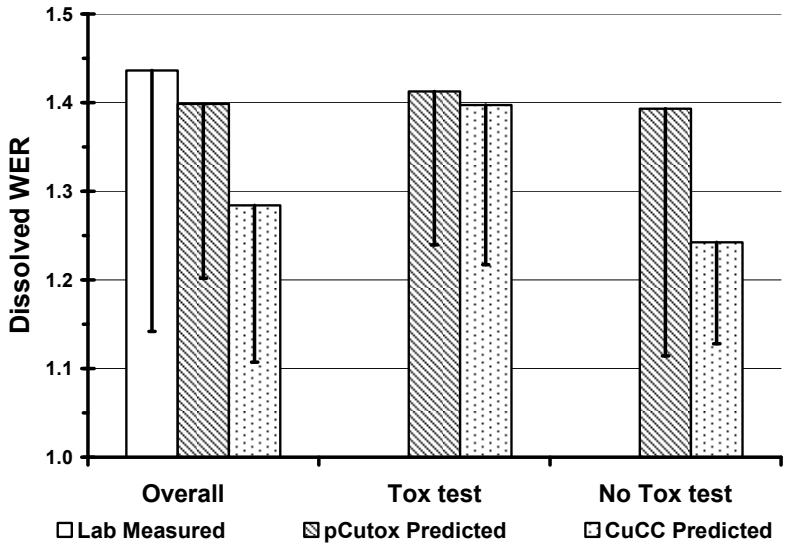

Figure 19. Comparison of the dissolved WERs estimated following the pCutox and the CuCC approaches to those measured in the laboratory following U.S. EPA-approved procedures. The bars represent the average and the line is one standard deviation. 'Tox test' indicates samples with actual toxicity measurements (n = 7), and 'No Tox test' indicates samples for which no toxicity testing was done (n = 19).

As Figure 19 shows, the average measured and predicted dissolved WERs are essentially identical when considering only those samples for which toxicity testing was also conducted, with average WERs of 1.44 ±0.29, 1.41 ±0.17, and 1.40 ±0.18 being observed for measured, pCu_{tox} predicted, and CuCC predicted WERs, respectively.

Similar averages (1.39 ±0.28) were obtained for samples for which no toxicity testing took place. This approach may be better in theory because it is directly based on the FIAM, and its application in natural settings, which has been already demonstrated (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Campbell, 1995, Ericksen et al., 2001; Rivera-Duarte et al., 2005) is further substantiated by these results.

In contrast to the values estimated following the pCu_{tox} approach, those estimated by the CuCC approach provide relatively lower values for the case when samples with no toxicity testing are used. Average values in the latter case are 1.24 ±0.11 range = 1.02 to 1.60) which are in the lower range of the values measured by toxicity testing (Figure 18). Although this may be associated with water quality characteristics unique to those samples resulting in slightly lower buffering, this approach may be considered weaker because of the relatively low number of samples used to generate the relationship between CuCC and lab measured EC50, and the low variability in the concentrations of metal binding ligands (e.g., DOC, TSS) used to generate that relationship. This approach could be strengthened by the use of data from several coastal waters, as is the case with the DOC approach (Arnold, Cotsifas, and Corneillie, 2006). In spite of these weaknesses, both pCu_{tox} and CuCC approaches show promise as methods for WER derivation.

ECOLOGICAL EFFECTS FROM COPPER

This study showed that Cu toxicity to *Mytilus* larvae in spiked samples from Sinclair and Dyes Inlets was less than predicted based on national WQC. *Mytilus* embryo-larval development was used as a test endpoint, because it has been shown to be the most sensitive test for chronic and acute effects from Cu in marine species (U.S. EPA, 1995b). Recently, studies have shown that exposure to Cu can cause effects to salmonid olfaction in freshwater (Hansen et al., 1999a, 1999b; Hansen, Lipton, and Welsh, 2002). An important environmental cue for species recognition, migration, reproduction, and predator avoidance in salmonids, olfaction inhibition has been linked to subchronic (behavioral) response caused by exposure to free uncomplexed Cu in freshwater systems (City of San Jose, 2005). Studies conducted at Northwest Fisheries Science Center (NWFSC) reported that juvenile salmonids were sensitive to Cu exposure in fresh water (sublethal olfactory impairment, Hecht et al. 2007) and that "water chemistry parameters [in fresh water were] less protective at the fish nose than at the fish gill against toxicity from dissolved copper" (McIntyre et al. 2008). However, the abundance and composition of ligands and organic matter (which bind to and detoxify metals) is different in salt water than in fresh water and it is unclear how Cu would affect older life stages of salmonids that have acclimatized to salt water (McIntyre et al. 2008).

The available laboratory studies were conducted in freshwaters that were low in hardness and organic carbon, which has significant impacts on the extrapolation of these data to natural marine systems. The most sensitive endpoint identified in a literature review conducted by the City of San Jose (2005), for example, indicated effects at concentrations of 0.8-µg dissolved Cu/L for Chinook salmon (Hansen et al., 1999a). That study, however, was run with well water diluted with deionized water resulting in a hardness of 25 mg/L $CaCO_3$. Additional water quality data provided by the authors allowed for the prediction of a free Cu concentration of 4.4×10^{-10} M, or a pCu of 9.4, using the BLM. This value is approximately 3 orders of magnitude higher than free Cu ion concentrations observed at numerous sampling locations in Sinclair and Dyes Inlets, as shown by our study.

Similarly, adjustment of the exceedingly low DOC concentration (0.03 mg/L) of the laboratory water used in the Hansen et al. (1999a) study to a more realistic value of 2 mg/L resulted in an increase of the effects level from 0.8 to 34 µg dissolved Cu/L (City of San Jose, 2005). These examples suggest that as with *Mytilus* embryo-larval development, olfactory inhibition in salmon is a function of exposure to free Cu ions, and therefore use of a WER-derived site-specific criterion would also be protective against potential effects to salmonids.

Recently, investigations were conducted by NWFSC to evaluate the effect of Cu exposure on olfactory impairment on Chinook salmon (*Onchorhynchus tshawytscha*) smolts in salt water (David Baldwin, NWFSC, personal communication). These experiments were conducted at NWFSC's Mukeltio Field Station using the flow-through seawater system to deliver site water from the Puget Sound for the experimental manipulations (dosing with Cu). In conjunction with this study SSC Pacific obtained and analyzed samples of seawater from the Mukilteo Field Station to provide data on saltwater chemistry and toxic effects of Cu to mussel (*Mytilus galloprovincialis*) embryos in the same source water used for the study of the effects of Cu on sublethal olfactory impairment in Chinook smolts (See Appendix F). The average dissolved and total Cu measured in samples collected from the seawater flow-through system of the Mukilteo Field Station were 0.15 µg/L (stdev 0.03, range 0.1 – 0.19 µg/L) and 0.18 µg/L (stdev 0.01, range 0.16 – 0.2 µg/L), respectively. The filtered Cu concentration (dissolved) accounted for about 87% of the total Cu present. Total and dissolved organic matter averaged about 1.5 mg/L suggesting that the organic carbon was present mainly in the dissolved phase. The samples also had relatively low concentrations of suspended solids averaging 13 mg/L (6 – 30 mg/L). The mussel embryo Normal Survival EC50s obtained from the measured dissolved Cu concentrations in samples from the Mukilteo Field Station ranged from 5.2 – 5.87 µg/L and the NOECs and LOECs were 3.7 and 5.3 µg/L dissolved Cu, respectively. As expected, the NOEC and LOEC for seawater samples from the Mukilteo Field Station were above the chronic and acute water quality standards for dissolved Cu. The EC50s obtained for the seawater from the Mukilteo Field Station were much lower than the EC50s determined for samples of ambient water (nearshore and marine) from Sinclair and Dyes Inlets, and were more than a factor of two below the regression reported by Arnold et al. (2006; EQU (1)), derived from WER studies conducted throughout North America. Seawater from the Mukilteo Field Station had very little binding capacity for Cu, consequently mussel embryos were very sensitive to Cu exposure (See Appendix F).

SUMMARY AND CONCLUSIONS

A toxicity assessment was conducted to evaluate the potential for ambient toxicity and the relative degree of copper bioavailability in surface water samples collected from five locations in Sinclair Inlet and one location in Dyes Inlet, adjacent to the PSNS&IMF in Puget Sound, Washington. Ambient site water samples were generally non-toxic to mussel (*Mytilus galloprovincialis*) embryos exposed in 48-hour embryo-larval development tests, and had dissolved copper concentrations substantially below ambient water quality criteria (AWQC; 3.1 µg dissolved Cu/L). Reduced normal survival of mussel embryos observed in two samples from the 9/27/2005 (Late Summer/Fall) sampling event was attributed to the presence of very high concentrations of a toxic dinoflagellate, *Gymnodinium splendens,* rather than toxicity associated with industrial discharges.

Copper additions to site and laboratory waters always resulted in toxic effects to developing mussel larvae. Resulting EC50 values based on the measured copper concentration in the site water toxicity tests were always higher than EC50s generated in laboratory water comparable to that used in AWQC development, indicating that the national WQC for copper is more protective than intended by the U.S. EPA at this site. As expected, total recoverable EC50 values were significantly correlated with both suspended solids, and dissolved EC50s were significantly correlated with DOC. Final dissolved and total recoverable water effect ratios (WERs) of 1.41 and 1.63 were calculated, respectively, following the determination of no statistical differences among individual WERs across sampling seasons and among the sampling locations within a sampling event. Based on these data, an adjustment of the national AWQC for dissolved copper by a factor of 1.41 would provide the level of protection intended by the U.S. EPA. Using this WER, acute and chronic site-specific dissolved copper criteria for Sinclair and Dyes Inlets, would be 6.8 and 4.4 µg/L, respectively.

This study also illustrated the utility of alternative strategies for deriving site-specific criteria for copper using either a DOC-toxicity model or copper comlexation capacity (CuCC), with both methods resulting in predicted final WERs within 5% of those measured using toxicity testing. The similarity among the measurements provides additional lines of evidence that support the results of the toxicity study, and suggest that less costly methods are available until a saltwater BLM for copper is successfully validated. The alternative strategies also allowed for predictions of WER values using larger data sets than those from the toxicity study, with the DOC-toxicity model yielding a final dissolved WER of 1.27, based on 117 DOC samples, and final WERs between 1.27 and 1.39 using a total of 26 samples for which pCu_{tox} and CuCC were used to predict WERs. None of the alternative measurements were statistically different from the final WERs derived using toxicity testing.

The very high sensitivity of *M. galloprovincialis* embryos to relatively low concentrations of dissolved copper makes it a relevant test endpoint on which to base a WER study. Recent studies indicating high copper sensitivity to salmonid endpoints (e.g., olfactory inhibition) were generally conducted in waters with characteristics appreciably different than those expected in Sinclair and Dyes Inlets. Samples of seawater obtained from the Mukilteo Field Station were analyzed for Cu, DOC, TSS, and mussel embryo toxicity to provide data on Cu bioavailability in the same site water used for the study of the effects on Cu on sublethal olfactory impairment in Chinook smolts in salt water (D.H. Baldwin, NWFSC, Seattle, WA, personal communication). The seawater from the Mukilteo Field Station had low dissolved Cu (average 0.15 µg/L; range 0.1 – 0.19 µg/L), DOC (average 1.5 mg/L, range 1.4 – 1.9 mg/L), and TSS (average 13 mg/L, range 6 – 30 mg/L) concentrations. The seawater from the Mukilteo Field Station had very little binding capacity for Cu, and consequently, mussel embryos were very sensitive to Cu exposure, resulting in mussel embryo Normal Survival EC50s that ranged from 5.2 to 5.87 µg/L and NOECs and LOECs of 4.1 and 5.8 µg/L dissolved Cu, respectively. As expected, the NOEC and LOEC for seawater samples from the Mukilteo Field Station were above the chronic and acute water quality standards for dissolved Cu.

Use of the Biotic Ligand Model to normalize toxic concentrations based on expected site-specific conditions (e.g., hardness, DOC concentrations) indicate that these endpoints would be adequately protected under a site-specific criterion based on the *M. galloprovincialis* results.

Because empirically derived WER data are available for Sinclair and Dyes Inlets as a result of this study, and WER studies are the current acceptable regulatory approach for site-specific criteria development, site-specific criteria for Cu discharges in Sinclair and Dyes Inlets are warranted in the development of NPDES permits in the local region.

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APPENDIX A. SITE WATER HANDLING SUMMARY FOR TOXICITY SAMPLES

Sampling S Date	Sample ID	ampling Time	Received at SSC-SD Date	Time	Temp (°C)	Test Initiation Date	Time	Elapsed time (hrs) Collection to Testing
3/31/2004	M3.1	805	4/1/2004	1045	5.0	4/1/2004	1700	33
	M3.3	838	4/1/2004	1045	5.0	4/1/2004	1700	32
	P3	905	4/1/2004	1045	5.0	4/1/2004	1700	32
2/9/2005	SN12	1015	2/10/2005	1220	7.1	2/11/2005	1215	50
	M3.1	1054	2/10/2005	1220	6.6	2/11/2005	1215	49
	M4	930	2/10/2005	1220	5.8	2/11/2005	1215	51
	P3	743	2/10/2005	1220	7.6	2/11/2005	1215	52
	M6	1201	2/10/2005	1220	5.8	2/11/2005	1215	48
9/27/2005	SN12	804	9/28/2005	1130	13.3	9/28/2005	1700	33
	M3.1	909	9/28/2005	1130	12.4	9/28/2005	1700	32
	M4	744	9/28/2005	1130	11.2	9/28/2005	1700	33
	P3	640	9/28/2005	1130	9.6	9/28/2005	1700	34
	M6	945	9/28/2005	1130	10.8	9/28/2005	1700	31

APPENDIX B. SUMMARY OF TARGET TOXICITY TEST CONDITIONS AND ACCEPTABILITY CRITERIA

Test conditions and acceptability criteria required by standardized toxicity test methods for bivalve embryo-larval development tests and those used in this study.

Criteria	ASTM 1999	USEPA 1995b	This Study
1. Test salinity (ppt)	18-32 ± 1	30 ± 2	30 ± 2
2. Test Temperature (deg. C)	16 ± 1	15 or 18 ± 1	15 ± 1
3. Light quality/intensity	Ambient lab levels	Ambient lab levels	Ambient lab levels
4. Photoperiod (hours)	16 h light: 8 h dark	16 h light: 8 h dark	16 h light: 8 h dark
5. Test chamber size (mL)	10-30	10-30	20
6. Test solution volume (mL)	10-30	10	10
7. Embryos/mL	15-30	15-30	15-30
8. Number of replicates/concentration	3	4	5
9. Dilution water	uncontaminated seawater	1 µm filtered natural seawater	0.45 µm natural seawater
10. Test duration (hours)	48	48-54	48
11. Test Endpoint	survival & normal shell dev.	survival & normal shell dev.	survival & normal shell dev.
12. Test Acceptability Criteria	1) ≥ 70% of introduced embryos must result in live larvae with completely developed shells in the controls 2) ≥ 70% normal shell dev in surviving controls	1) control survival must be ≥ 50% 2) ≥ 90% normal shell dev. in surviving controls 3) % MSD < 25%	1) ≥ 70% of introduced embryos must result in live larvae with completely developed shells in the controls 2) ≥ 70% normal shell dev in surviving controls
13. Broodstock geographical area reported and consistent	yes	yes	yes
14. Initiation of test after fertilization	within 4 h	within 4 h	within 4 h
15. Sample holding time (h)		< 36	< 96[1]
16. Lab water TSS/TOC requirements	< 5 mg/L		< 5 mg/L
17. D.O., salinity, temp., pH measured	yes	yes	yes
18. D.O. level/% saturation	60-100% sat	> 4.0 mg/L	> 4.0 mg/L

[1]As required by USEPA 2001 (Streamlined Water-Effect Ratio Procedure for Discharges of Copper)

APPENDIX C. WATER QUALITY FROM TOXICITY TESTS

Water quality measurements from Spring sampling event (3/31/2004)

Sample ID	Nominal [Cu] µg/l	pH (SU)	D.O. (mg/L)	Temperature (°C)	Salinity (‰)
LW	0	8.01	nd	16.0	29
	4.1	8.10	nd	16.2	29
	8.4	8.01	nd	16.2	29
	17.2	8.01	nd	16.1	29
M3.1	0	7.93	7.6	15.8	30
	2.9	7.95	nd	15.9	30
	8.4	7.95	nd	15.9	30
	50	7.99	nd	15.8	29
M3.3	0	7.94	7.6	15.8	29
	2.9	7.95	nd	16.1	29
	8.4	7.95	nd	15.8	29
	50	7.95	nd	16.1	29
P3	0	7.89	7.5	15.9	30
	2.9	7.91	nd	15.7	29
	8.4	7.91	nd	15.7	30
	17.2	7.91	nd	15.7	29
RT	0	8.00	6.0	15.8	34
	2.9	7.95	nd	15.7	34
	8.4	8.00	nd	15.8	34
	17.2	7.99	nd	15.7	34

nd=not determined

Note: Water quality for this data set measured at test initiation only.

Winter (2/9/2005) sampling event water quality summary from toxicity tests.

Sample ID	Nominal [Cu] (µg/l)	pH (SU)			D.O. (mg/l)			Temperature (°C)			Salinity (‰)		
		Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
LW	0	7.9	7.7	7.8	7.6	6.8	7.3	15.8	15.1	15.4	28.2	27.8	28.0
	2.9	7.9	7.7	7.8	7.7	6.9	7.4	15.7	15.0	15.4	29.0	28.4	28.6
	4.1	7.9	7.8	7.8	7.8	6.9	7.4	15.8	15.1	15.4	28.3	28.0	28.1
	5.9	7.9	7.8	7.8	8.1	6.8	7.6	15.8	15.0	15.4	28.4	27.6	28.0
	8.4	7.9	7.7	7.8	7.9	6.9	7.5	15.9	15.0	15.4	28.2	28.0	28.1
	12	7.9	7.7	7.8	8.0	7.0	7.5	16.0	15.3	15.5	28.3	28.1	28.2
	17.2	7.9	7.7	7.8	8.0	7.2	7.5	15.8	15.0	15.4	28.4	28.0	28.2
SN12	0	7.8	7.6	7.7	7.7	6.9	7.2	15.8	15.4	15.6	28.2	27.5	27.8
	2.9	7.8	7.6	7.7	7.7	7.1	7.4	15.7	15.4	15.5	28.1	28.0	28.0
	4.1	7.8	7.6	7.7	7.6	7.2	7.3	15.6	15.4	15.5	28.5	28.2	28.3
	5.9	7.8	7.6	7.7	7.7	7.2	7.4	15.5	15.1	15.3	28.6	28.3	28.4
	8.4	7.8	7.6	7.7	7.8	7.0	7.3	15.8	15.3	15.5	28.4	28.2	28.3
	12	7.8	7.6	7.7	7.8	7.2	7.4	15.4	15.0	15.2	28.5	28.1	28.4
	17.2	7.8	7.6	7.7	7.9	7.3	7.5	15.4	15.2	15.3	28.6	27.7	28.2
M3.1	0	7.8	7.7	7.7	7.8	7.1	7.4	15.7	15.3	15.5	28.5	28.1	28.3
	2.9	7.8	7.6	7.7	7.8	7.2	7.4	15.7	15.4	15.5	28.6	28.3	28.5
	4.1	7.8	7.6	7.7	7.8	6.9	7.3	15.6	15.4	15.5	28.8	28.6	28.7
	5.9	7.8	7.6	7.7	7.7	7.0	7.3	15.6	15.3	15.5	29.2	29.0	29.1
	8.4	7.8	7.6	7.7	7.6	7.3	7.4	15.5	15.1	15.3	28.4	27.3	27.9
	12	7.8	7.6	7.7	7.7	7.3	7.5	15.5	15.4	15.5	28.9	28.6	28.8
	17.2	7.8	7.6	7.7	7.8	7.3	7.6	15.5	15.3	15.4	29.0	28.2	28.7
M4	0	7.8	7.6	7.7	7.7	7.2	7.5	15.7	15.5	15.6	28.6	27.8	28.3
	2.9	7.8	7.6	7.7	7.7	7.2	7.5	15.7	15.6	15.6	28.7	28.3	28.5
	4.1	7.8	7.6	7.7	7.9	7.4	7.6	15.5	15.4	15.4	28.7	27.7	28.3
	5.9	7.8	7.6	7.7	7.9	7.4	7.6	15.6	15.3	15.4	28.6	26.4	27.7
	8.4	7.8	7.6	7.7	8.0	7.5	7.7	15.6	15.2	15.4	28.2	27.3	27.8
	12	7.8	7.6	7.7	8.0	7.4	7.6	15.7	15.2	15.5	28.1	27.8	28.0
	17.2	7.8	7.6	7.7	8.0	7.2	7.5	15.6	15.1	15.4	28.3	27.6	28.0
P3	0	7.8	7.6	7.7	7.8	7.1	7.4	15.7	15.4	15.6	28.5	27.9	28.3
	2.9	7.8	7.6	7.7	7.8	7.1	7.4	15.7	15.3	15.5	29.0	28.5	28.8
	4.1	7.8	7.6	7.7	7.8	7.0	7.4	15.6	15.6	15.6	29.0	28.5	28.8
	5.9	7.8	7.6	7.7	7.9	7.0	7.5	15.6	15.5	15.6	29.0	28.2	28.6
	8.4	7.8	7.6	7.7	7.9	7.2	7.6	15.5	15.4	15.5	29.4	28.9	29.1
	12	7.8	7.6	7.7	7.9	7.0	7.4	15.5	15.3	15.4	29.0	28.4	28.6
	17.2	7.8	7.6	7.7	7.8	7.2	7.4	15.6	15.4	15.5	29.3	29.0	29.1
M6	0	7.8	7.7	7.7	7.7	6.8	7.3	15.7	15.2	15.5	29.4	28.6	29.0
	2.9	7.8	7.7	7.7	7.8	7.0	7.4	15.8	15.3	15.6	29.7	28.8	29.2
	4.1	7.8	7.7	7.7	7.8	7.4	7.6	15.7	15.4	15.6	29.1	26.9	28.0
	5.9	7.8	7.6	7.7	7.7	7.4	7.6	15.8	15.3	15.6	29.1	28.0	28.4
	8.4	7.8	7.7	7.7	7.9	7.6	7.7	15.5	15.4	15.5	29.2	28.7	29.0
	12	7.8	7.6	7.7	7.9	7.6	7.7	15.5	15.3	15.4	28.6	28.2	28.4
	17.2	7.8	7.7	7.7	7.9	7.3	7.6	15.5	15.4	15.4	29.3	29.0	29.2

Summer/Fall (9/27/2005) sampling event water quality summary from toxicity tests.

Sample ID	Nominal [Cu] (µg/l)	pH (SU)			D.O. (mg/l)			Temperature (°C)			Salinity (‰)		
		Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
LW	0	8.0	7.9	7.9	7.9	6.9	7.5	15.4	14.6	15.1	30.1	29.3	29.6
	2.9	8.0	7.9	7.9	7.9	7.8	7.9	15.4	15.2	15.3	30.0	30.0	30.0
	4.1	7.9	7.8	7.9	8.0	7.7	7.9	15.7	14.8	15.3	30.1	29.7	29.9
	5.9	7.9	7.8	7.9	7.9	7.8	7.9	15.5	14.8	15.2	30.1	30.0	30.0
	8.4	7.9	7.8	7.9	8.0	7.7	7.9	15.5	14.8	15.2	30.1	30.0	30.0
	12	7.9	7.8	7.9	7.9	7.8	7.9	15.6	14.8	15.3	30.1	30.0	30.0
	17.2	7.9	7.8	7.9	7.9	7.9	7.9	15.4	14.4	14.9	30.1	30.0	30.1
	24	7.9	7.8	7.9	8.0	7.9	7.9	15.4	14.8	15.2	30.2	30.1	30.1
SN12	0	8.1	7.9	8.0	8.0	7.8	7.9	15.2	14.6	14.9	30.2	30.0	30.1
	2.9	8.1	8.0	8.1	8.1	7.7	7.9	15.2	14.9	15.1	30.2	30.0	30.1
	4.1	8.1	8.0	8.1	8.0	7.8	7.9	15.5	14.9	15.2	30.2	30.1	30.2
	5.9	8.1	8.0	8.1	8.1	7.8	7.9	15.1	14.8	15.0	30.3	30.1	30.2
	8.4	8.1	8.0	8.1	8.1	7.9	8.0	15.4	14.8	15.1	30.2	30.0	30.1
	12	8.1	8.0	8.1	8.0	7.9	8.0	15.4	14.7	15.1	30.2	30.0	30.1
	17.2	8.1	8.0	8.1	8.0	7.8	7.9	15.1	14.6	14.8	30.2	30.0	30.1
	24	8.1	8.0	8.1	8.0	7.9	7.9	15.4	14.8	15.1	30.3	30.0	30.2
	35	8.1	8.0	8.0	8.0	7.9	7.9	15.0	14.6	14.8	30.3	30.1	30.2
M3.1	0	8.2	8.1	8.2	7.8	7.4	7.6	16.0	14.8	15.3	31.2	30.0	30.5
	2.9	8.1	8.0	8.1	8.1	7.4	7.8	16.0	15.2	15.5	31.1	31.0	31.1
	4.1	8.1	8.0	8.1	8.1	7.7	7.9	15.7	15.2	15.4	31.1	31.0	31.0
	5.9	8.0	8.0	8.0	7.9	7.7	7.8	15.7	14.9	15.3	31.2	31.0	31.1
	8.4	8.0	8.0	8.0	8.0	7.7	7.9	15.7	14.6	15.2	31.2	31.0	31.1
	12	8.0	8.0	8.0	8.1	7.6	7.9	15.7	15.1	15.3	31.2	31.0	31.1
	17.2	8.0	7.9	8.0	8.0	7.7	7.9	15.7	14.9	15.3	31.2	31.0	31.1
	24	8.0	7.9	7.9	8.1	7.8	7.9	15.8	15.4	15.5	31.2	31.0	31.1
	35	8.0	7.9	8.0	8.0	7.4	7.8	15.8	14.4	14.9	31.2	31.0	31.1
M4	0	7.9	7.8	7.9	7.7	7.5	7.6	16.0	14.7	15.2	29.7	29.4	29.6
	2.9	7.9	7.8	7.8	7.6	7.5	7.6	15.8	14.8	15.4	29.9	29.7	29.8
	4.1	7.9	7.9	7.9	7.9	7.5	7.7	16.0	15.1	15.5	30.2	30.0	30.1
	5.9	7.9	7.9	7.9	7.6	7.4	7.5	15.8	14.8	15.3	30.5	30.2	30.3
	8.4	7.9	7.8	7.9	7.6	7.3	7.5	15.7	15.2	15.5	30.4	30.2	30.3
	12	7.9	7.8	7.9	7.6	7.3	7.4	15.8	14.7	15.1	30.4	30.1	30.3
	17.2	7.9	7.8	7.8	7.6	7.3	7.4	15.8	14.5	15.2	30.4	30.1	30.3
	24	7.9	7.8	7.8	7.4	7.3	7.3	15.8	14.8	15.4	30.4	30.2	30.3
	35	7.8	7.7	7.8	6.9	5.5	6.3	15.9	14.5	15.0	30.5	30.2	30.4
	50	7.8	7.6	7.7	6.8	4.6	6.0	16.0	14.6	15.1	30.6	30.3	30.5

Summer/Fall (9/27/2005) sampling event water quality summary from toxicity tests.

Sample ID	Nominal [Cu] (µg/l)	pH (SU)			D.O. (mg/l)			Temperature (°C)			Salinity (‰)		
		Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
P3	0	7.9	7.8	7.9	8.0	7.6	7.8	15.5	14.7	15.0	29.0	28.7	28.8
	2.9	7.9	7.9	7.9	8.0	7.6	7.8	15.8	15.5	15.7	29.0	28.8	28.9
	4.1	7.9	7.9	7.9	8.0	7.7	7.9	15.5	15.0	15.2	29.0	28.9	29.0
	5.9	7.9	7.9	7.9	7.9	7.6	7.7	15.5	15.2	15.4	29.0	28.9	29.0
	8.4	7.9	7.9	7.9	8.1	7.6	7.9	15.2	15.0	15.1	29.0	28.7	28.9
	12	7.9	7.9	7.9	8.1	8.0	8.0	15.1	14.6	14.8	29.0	28.8	28.9
	17.2	7.9	7.9	7.9	8.1	7.7	8.0	15.5	15.2	15.4	29.1	28.9	29.0
	24	7.9	7.9	7.9	8.1	7.7	7.9	15.4	15.2	15.3	29.1	28.9	29.0
	35	7.9	7.9	7.9	8.0	7.7	7.9	15.3	14.7	14.9	29.1	29.0	29.0
M6	0	7.9	7.8	7.9	7.8	7.7	7.8	16.0	14.6	15.3	30.9	30.3	30.6
	2.9	7.9	7.9	7.9	7.8	7.7	7.7	16.0	15.4	15.8	30.9	30.6	30.7
	4.1	7.9	7.9	7.9	7.9	7.8	7.8	15.8	15.6	15.7	30.9	30.7	30.8
	5.9	7.9	7.9	7.9	8.0	7.7	7.8	15.7	15.3	15.5	30.9	30.7	30.8
	8.4	7.9	7.9	7.9	7.9	7.7	7.8	15.8	14.9	15.4	31.0	30.8	30.9
	12	7.9	7.9	7.9	7.9	7.7	7.8	15.7	15.2	15.4	31.0	30.8	30.9
	17.2	7.9	7.9	7.9	7.9	7.6	7.8	15.8	15.4	15.6	31.0	30.8	30.9
	24	7.9	7.8	7.9	8.0	7.7	7.9	15.8	15.2	15.4	31.0	30.8	30.9
	35	7.9	7.8	7.9	8.0	7.7	7.9	15.8	14.6	15.2	31.1	30.8	31.0

APPENDIX D. TOXICITY TEST DATA

The following tables show the toxicity test results from each of the toxicity tests conducted for the three sampling events. The initial number of larvae from each vial is an estimate calculated from the mean of 5 initial density vials that were preserved with formalin immediately after addition of embryos at the start of the test (Table D1). The normal survival endpoint was then calculated as the ratio of the number of normal D-shaped larvae counted in each vial at the end of the test to the initial density. Unless otherwise noted, dashed lines instead of numerical values indicate that abnormal larvae were not enumerated because no normal larvae were present in the vial, rendering the measurement unmeaningful.

Figure D-1. Raw values, mean, and standard deviation of initial embryo density vials used for larval survival assessment. Dash indicates no data available for that replicate.

Replicate	Event #1 (3/31/2004)	Event #2 (2/9/2005)	Event #3 (9/27/2005)
A	161	237	181
B	142	214	180
C	138	211	176
D	158	204	181
E	-	246	197
Mean	150	222	183
S.D.	11.4	18.1	8.1

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004
Sample ID: LW

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	150	143	2	145	99	95
0	b	150	144	3	147	98	96
0	c	150	123	2	125	98	82
0	d	150	176	2	178	99	117
4.0	a	150	151	2	153	99	101
4.0	b	150	154	5	159	97	103
4.0	c	150	137	2	139	99	91
4.0	d	150	136	2	138	99	91
5.7	a	150	132	5	137	96	88
5.7	b	150	123	5	128	96	82
5.7	c	150	141	3	144	98	94
5.7	d	150	139	2	141	99	93
8.3	a	150	121	23	144	84	81
8.3	b	150	124	9	133	93	83
8.3	c	150	124	13	137	91	83
8.3	d	150	141	4	145	97	94
11.8	a	150	38	123	161	24	25
11.8	b	150	15	118	133	11	10
11.8	c	150	16	115	131	12	11
11.8	d	150	2	141	143	1	1
17.0	a	150	0	139	139	0	0
17.0	b	150	0	151	151	0	0
17.0	c	150	0	147	147	0	0
17.0	d	150	0	141	141	0	0

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004
Sample ID: RT

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	150	130	4	134	97	87
0	b	150	136	1	137	99	91
0	c	150	134	2	136	99	89
0	d	150	131	1	132	99	87
4.0	a	150	146	1	147	99	97
4.0	b	150	111	1	112	99	74
4.0	c	150	144	2	146	99	96
4.0	d	150	121	5	126	96	81
5.7	a	150	141	1	142	99	94
5.7	b	150	150	3	153	98	100
5.7	c	150	155	2	157	99	103
5.7	d	150	136	2	138	99	91
8.3	a	150	119	8	127	94	79
8.3	b	150	140	11	151	93	93
8.3	c	150	161	4	165	98	107
8.3	d	150	136	4	140	97	91
11.8	a	150	23	108	131	18	15
11.8	b	150	65	77	142	46	43
11.8	c	150	24	97	121	20	16
11.8	d	150	66	92	158	42	44
17.0	a	150	0	139	139	0	0
17.0	b	150	1	120	121	1	1
17.0	c	150	0	143	143	0	0
17.0	d	150	0	141	141	0	0

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004
Sample ID: M3.1

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	150	126	1	127	99	84
0	b	150	145	4	149	97	97
0	c	150	110	2	112	98	73
0	d	150	142	5	147	97	95
4.0	a	150	126	4	130	97	84
4.0	b	150	147	2	149	99	98
4.0	c	150	114	2	116	98	76
4.0	d	150	136	4	140	97	91
5.7	a	150	129	2	131	98	86
5.7	b	150	78	6	84	93	52
5.7	c	150	161	3	164	98	107
5.7	d	150	124	2	126	98	83
8.3	a	150	156	6	162	96	104
8.3	b	150	152	3	155	98	101
8.3	c	150	138	5	143	97	92
8.3	d	150	161	3	164	98	107
11.8	a	150	119	11	130	92	79
11.8	b	150	132	12	144	92	88
11.8	c	150	139	4	143	97	93
11.8	d	150	139	10	149	93	93
17.0	a	150	26	119	145	18	17
17.0	b	150	5	74	79	6	3
17.0	c	150	86	62	148	58	57
17.0	d	150	34	128	162	21	23
24.6	a	150	4	120	124	3	3
24.6	b	150	8	150	158	5	5
24.6	c	150	6	152	158	4	4
24.6	d	150	7	134	141	5	5

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004
Sample ID: P3

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	150	159	0	159	100	106
0	b	150	149	2	151	99	99
0	c	150	154	4	158	97	103
0	d	150	138	5	143	97	92
4.0	a	150	154	1	155	99	103
4.0	b	150	140	2	142	99	93
4.0	c	150	133	5	138	96	89
4.0	d	150	150	2	152	99	100
5.7	a	150	157	6	163	96	105
5.7	b	150	152	3	155	98	101
5.7	c	150	155	2	157	99	103
5.7	d	150	169	3	172	98	113
8.3	a	150	160	6	166	96	107
8.3	b	150	136	1	137	99	91
8.3	c	150	147	3	150	98	98
8.3	d	150	130	1	131	99	87
11.8	a	150	109	35	144	76	73
11.8	b	150	65	73	138	47	43
11.8	c	150	109	35	144	76	73
11.8	d	150	111	32	143	78	74
17.0	a	150	50	102	152	33	33
17.0	b	150	4	138	142	3	3
17.0	c	150	5	147	152	3	3
17.0	d	150	3	104	107	3	2
24.6	a	150	0	111	111	0	0
24.6	b	150	1	129	130	1	1
24.6	c	150	0	140	140	0	0
24.6	d	150	0	135	135	0	0

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004
Sample ID: M3.3

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	150	139	1	140	99	93
0	b	150	146	5	151	97	97
0	c	150	148	0	148	100	99
0	d	150	118	6	124	95	79
4.0	a	150	157	1	158	99	105
4.0	b	150	123	6	129	95	82
4.0	c	150	136	6	142	96	91
4.0	d	150	150	5	155	97	100
5.7	a	150	145	2	147	99	97
5.7	b	150	153	8	161	95	102
5.7	c	150	162	8	170	95	108
5.7	d	150	128	3	131	98	85
8.3	a	150	113	1	114	99	75
8.3	b	150	144	4	148	97	96
8.3	c	150	118	2	120	98	79
8.3	d	150	150	1	151	99	100
11.8	a	150	140	11	151	93	93
11.8	b	150	155	6	161	96	103
11.8	c	150	146	6	152	96	97
11.8	d	150	145	6	151	96	97
17.0	a	150	53	109	162	33	35
17.0	b	150	95	42	137	69	63
17.0	c	150	44	87	131	34	29
17.0	d	150	47	72	119	39	31
24.6	a	150	0	141	141	0	0
24.6	b	150	0	133	133	0	0
24.6	c	150	0	136	156	0	0
24.6	d	150	0	131	131	0	0

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: LW

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	160	4	164	98	72
0	b	222	222	4	226	98	100
0	c	222	236	6	242	98	106
0	d	222	233	9	242	96	105
0	e	222	257	3	260	99	116
2.9	a	222	231	5	236	98	104
2.9	b	222	207	4	211	98	93
2.9	c	222	214	1	215	100	96
2.9	d	222	201	3	204	99	91
2.9	e	222	228	2	230	99	103
4.1	a	222	214	8	222	96	96
4.1	b	222	205	21	226	91	92
4.1	c	222	111	85	196	57	50
4.1	d	222	83	134	217	38	37
4.1	e	222	0	205	205	0	0
5.9	a	222	125	101	226	55	56
5.9	b	222	125	94	219	57	56
5.9	c	222	132	106	238	55	59
5.9	d	222	114	125	239	48	51
5.9	e	222	64	142	206	31	29
8.4	a	222	23	187	210	11	10
8.4	b	222	24	173	197	12	11
8.4	c	222	4	210	214	2	2
8.4	d	222	32	170	202	16	14
8.4	e	222	21	200	221	10	9
12.0	a	222	0	195	195	0	0
12.0	b	222	1	246	247	0	0
12.0	c	222	1	221	222	0	0
12.0	d	222	1	218	219	0	0
12.0	e	222	0	219	219	0	0
17.2	a	222	0	210	210	0	0
17.2	b	222	0	187	187	0	0
17.2	c	222	0	178	178	0	0
17.2	d	222	0	215	215	0	0
17.2	e	222	0	198	198	0	0

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: SN12

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	232	3	235	99	105
0	b	222	206	3	209	99	93
0	c	222	219	4	223	98	99
0	d	222	219	1	220	100	99
0	e	222	238	2	240	99	107
2.9	a	222	199	9	208	96	90
2.9	b	222	206	4	210	98	93
2.9	c	222	235	6	241	98	106
2.9	d	222	206	5	211	98	93
2.9	e	222	224	4	228	98	101
4.1	a	222	234	4	238	98	105
4.1	b	222	164	6	170	96	74
4.1	c	222	193	8	201	96	87
4.1	d	222	160	8	168	95	72
4.1	e	222	230	2	232	99	104
5.9	a	222	202	3	205	99	91
5.9	b	222	219	1	220	100	99
5.9	c	222	223	5	228	98	100
5.9	d	222	231	9	240	96	104
5.9	e	222	211	9	220	96	95
8.4	a	222	56	205	261	21	25
8.4	b	222	56	166	222	25	25
8.4	c	222	129	100	229	56	58
8.4	d	222	93	132	225	41	42
8.4	e	222	78	150	228	34	35
12.0	a	222	19	251	270	7	9
12.0	b	222	0	229	229	0	0
12.0	c	222	4	197	201	2	2
12.0	d	222	14	208	222	6	6
12.0	e	222	13	225	238	5	6
17.2	a	222	0	210	210	0	0
17.2	b	222	0	189	189	0	0
17.2	c	222	0	220	220	0	0
17.2	d	222	0	232	232	0	0
17.2	e	222	0	211	211	0	0

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: M3.1

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	234	4	238	98	105
0	b	222	221	4	225	98	100
0	c	222	211	3	214	99	95
0	d	222	222	2	224	99	100
0	e	222	260	7	267	97	117
2.9	a	222	193	3	196	98	87
2.9	b	222	208	0	208	100	94
2.9	c	222	203	2	205	99	91
2.9	d	222	194	12	206	94	87
2.9	e	222	209	3	212	99	94
4.1	a	222	199	3	202	99	90
4.1	b	222	211	0	211	100	95
4.1	c	222	259	0	259	100	117
4.1	d	222	221	4	225	98	100
4.1	e	222	229	5	234	98	103
5.9	a	222	216	4	220	98	97
5.9	b	222	219	6	225	97	99
5.9	c	222	236	5	241	98	106
5.9	d	222	219	6	225	97	99
5.9	e	222	211	8	219	96	95
8.4	a	222	180	21	201	90	81
8.4	b	222	194	20	214	91	87
8.4	c	222	217	9	226	96	98
8.4	d	222	185	15	200	93	83
8.4	e	222	200	18	218	92	90
12.0	a	222	8	201	209	4	4
12.0	b	222	28	196	224	13	13
12.0	c	222	52	152	204	25	23
12.0	d	222	31	219	250	12	14
12.0	e	222	21	197	218	10	9
17.2	a	222	2	239	241	1	1
17.2	b	222	1	214	215	0	0
17.2	c	222	1	214	215	0	0
17.2	d	222	0	146	146	0	0
17.2	e	222	1	249	250	0	0

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: M4

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	229	3	232	99	103
0	b	222	203	1	204	100	91
0	c	222	207	2	209	99	93
0	d	222	236	3	239	99	106
0	e	222	-	-	-	-	-
2.9	a	222	233	3	236	99	105
2.9	b	222	243	7	250	97	109
2.9	c	222	200	1	201	100	90
2.9	d	222	215	3	218	99	97
2.9	e	222	230	4	234	98	104
4.1	a	222	242	6	248	98	109
4.1	b	222	250	4	254	98	113
4.1	c	222	192	6	198	97	86
4.1	d	222	219	6	225	97	99
4.1	e	222	232	5	237	98	105
5.9	a	222	209	3	212	99	94
5.9	b	222	210	4	214	98	95
5.9	c	222	205	3	208	99	92
5.9	d	222	237	11	248	96	107
5.9	e	222	231	3	234	99	104
8.4	a	222	183	26	209	88	82
8.4	b	222	194	7	201	97	87
8.4	c	222	162	54	216	75	73
8.4	d	222	183	30	213	86	82
8.4	e	222	190	26	216	88	86
12.0	a	222	37	199	236	16	17
12.0	b	222	14	194	208	7	6
12.0	c	222	1	193	194	1	0
12.0	d	222	4	193	197	2	2
12.0	e	222	9	205	214	4	4
17.2	a	222	0	234	234	0	0
17.2	b	222	0	205	205	0	0
17.2	c	222	0	210	210	0	0
17.2	d	222	0	212	212	0	0
17.2	e	222	0	224	224	0	0

Dash indicates no data. Embryos not added.

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: P3

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	203	3	206	99	91
0	b	222	240	5	245	98	108
0	c	222	200	6	206	97	90
0	d	222	192	7	199	96	86
0	e	222	213	9	222	96	96
2.9	a	222	231	4	235	98	104
2.9	b	222	221	5	226	98	100
2.9	c	222	208	8	216	96	94
2.9	d	222	243	7	250	97	109
2.9	e	222	243	3	246	99	109
4.1	a	222	201	4	205	98	91
4.1	b	222	218	10	228	96	98
4.1	c	222	219	16	235	93	99
4.1	d	222	228	4	232	98	103
4.1	e	222	202	11	213	95	91
5.9	a	222	198	20	218	91	89
5.9	b	222	196	8	204	96	88
5.9	c	222	182	22	204	89	82
5.9	d	222	235	20	255	92	106
5.9	e	222	220	8	228	96	99
8.4	a	222	156	11	167	93	70
8.4	b	222	77	145	222	35	35
8.4	c	222	53	145	198	27	24
8.4	d	222	46	170	216	21	21
8.4	e	222	81	151	232	35	36
12.0	a	222	1	229	230	0	0
12.0	b	222	1	222	223	0	0
12.0	c	222	0	205	205	0	0
12.0	d	222	0	194	194	0	0
12.0	e	222	0	221	221	0	0
17.2	a	222	0	200	200	0	0
17.2	b	222	0	200	200	0	0
17.2	c	222	0	200	200	0	0
17.2	d	222	0	200	200	0	0
17.2	e	222	0	200	200	0	0

Sampling Event #2: 2/9/05
Test Initiation Date: 2/11/05
Sample ID: M6

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	222	217	2	219	99	98
0	b	222	221	5	226	98	100
0	c	222	201	3	204	99	91
0	d	222	218	1	219	100	98
0	e	222	221	1	222	100	100
2.9	a	222	222	3	225	99	100
2.9	b	222	208	0	208	100	94
2.9	c	222	208	4	212	98	94
2.9	d	222	223	4	227	98	100
2.9	e	222	192	2	194	99	86
4.1	a	222	209	2	211	99	94
4.1	b	222	218	4	222	98	98
4.1	c	222	228	0	228	100	103
4.1	d	222	192	3	195	98	86
4.1	e	222	217	5	222	98	98
5.9	a	222	225	3	228	99	101
5.9	b	222	204	4	208	98	92
5.9	c	222	220	1	221	100	99
5.9	d	222	202	4	206	98	91
5.9	e	222	214	7	221	97	96
8.4	a	222	213	13	226	94	96
8.4	b	222	203	29	232	88	91
8.4	c	222	174	36	210	83	78
8.4	d	222	137	74	211	65	62
8.4	e	222	176	32	208	85	79
12.0	a	222	41	203	244	17	18
12.0	b	222	15	193	208	7	7
12.0	c	222	29	201	230	13	13
12.0	d	222	23	185	208	11	10
12.0	e	222	39	204	243	16	18
17.2	a	222	10	236	246	4	5
17.2	b	222	0	213	213	0	0
17.2	c	222	1	216	217	0	0
17.2	d	222	0	121	121	0	0
17.2	e	222	0	208	208	0	0

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: LW

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	159	7	166	96	87
0	b	183	175	7	182	96	96
0	c	183	168	9	177	95	92
0	d	183	145	8	153	95	79
0	e	183	165	9	174	95	90
2.9	a	183	151	15	166	91	83
2.9	b	183	169	11	180	94	92
2.9	c	183	199	4	203	98	109
2.9	d	183	193	17	210	92	105
2.9	e	183	191	11	202	95	104
4.1	a	183	140	48	188	74	77
4.1	b	183	126	51	177	71	69
4.1	c	183	116	66	182	64	63
4.1	d	183	135	37	172	78	74
4.1	e	183	139	45	184	76	76
5.9	a	183	24	161	185	13	13
5.9	b	183	26	141	167	16	14
5.9	c	183	2	169	171	1	1
5.9	d	183	18	159	177	10	10
5.9	e	183	4	140	144	3	2
8.4	a	183	0	-	0	0	0
8.4	b	183	0	-	0	0	0
8.4	c	183	0	-	0	0	0
8.4	d	183	0	-	0	0	0
8.4	e	183	0	-	0	0	0
12.0	a	183	0	-	0	0	0
12.0	b	183	0	-	0	0	0
12.0	c	183	0	-	0	0	0
12.0	d	183	0	-	0	0	0
12.0	e	183	0	-	0	0	0
17.2	a	183	0	-	0	0	0
17.2	b	183	0	-	0	0	0
17.2	c	183	0	-	0	0	0
17.2	d	183	0	-	0	0	0
17.2	e	183	0	-	0	0	0
24.0	a	183	0	-	0	0	0
24.0	b	183	0	-	0	0	0
24.0	c	183	0	-	0	0	0
24.0	d	183	0	-	0	0	0
24.0	e	183	0	-	0	0	0

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: SN12

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	187	5	192	97	102
0	b	183	181	12	193	94	99
0	c	183	163	8	171	95	89
0	d	183	160	2	162	99	87
0	e	183	161	5	166	97	88
2.9	a	183	152	7	159	96	83
2.9	b	183	147	4	151	97	80
2.9	c	183	145	6	151	96	79
2.9	d	183	181	6	187	97	99
2.9	e	183	160	6	166	96	87
4.1	a	183	180	9	189	95	98
4.1	b	183	172	8	180	96	94
4.1	c	183	142	9	151	94	78
4.1	d	183	174	8	182	96	95
4.1	e	183	181	14	195	93	99
5.9	a	183	137	42	179	77	75
5.9	b	183	76	4	80	95	42
5.9	c	183	168	18	186	90	92
5.9	d	183	146	19	165	88	80
5.9	e	183	165	13	178	93	90
8.4	a	183	131	57	188	70	72
8.4	b	183	168	19	187	90	92
8.4	c	183	152	38	190	80	83
8.4	d	183	96	65	161	60	52
8.4	e	183	161	18	179	90	88
12.0	a	183	5	118	123	4	3
12.0	b	183	8	123	131	6	4
12.0	c	183	3	155	158	2	2
12.0	d	183	1	96	97	1	1
12.0	e	183	9	121	130	7	5
17.2	a	183	0	-	0	0	0
17.2	b	183	0	-	0	0	0
17.2	c	183	0	-	0	0	0
17.2	d	183	0	-	0	0	0
17.2	e	183	0	-	0	0	0
24.0	a	183	0	-	0	0	0
24.0	b	183	0	-	0	0	0
24.0	c	183	0	-	0	0	0
24.0	d	183	0	-	0	0	0
24.0	e	183	0	-	0	0	0
35.0	a	183	0	-	0	0	0
35.0	b	183	0	-	0	0	0
35.0	c	183	0	-	0	0	0
35.0	d	183	0	-	0	0	0
35.0	e	183	0	-	0	0	0

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: M3.1

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	148	9	157	94	102
0	b	183	179	13	192	93	99
0	c	183	156	5	161	97	89
0	d	183	155	13	168	92	87
0	e	183	148	4	152	97	88
2.9	a	183	159	13	172	92	83
2.9	b	183	148	11	159	93	80
2.9	c	183	164	9	173	95	79
2.9	d^	183	23	7	30	77	99
2.9	e	183	177	11	188	94	87
4.1	a	183	173	10	183	95	98
4.1	b	183	159	5	164	97	94
4.1	c	183	156	8	164	95	78
4.1	d	183	142	11	153	93	95
4.1	e	183	144	17	161	89	99
5.9	a	183	169	8	177	95	75
5.9	b	183	179	7	186	96	42
5.9	c	183	159	10	169	94	92
5.9	d	183	131	6	137	96	80
5.9	e	183	153	7	160	96	90
8.4	a	183	132	49	181	73	72
8.4	b	183	133	44	177	75	92
8.4	c	183	123	45	168	73	83
8.4	d	183	126	52	178	71	52
8.4	e	183	116	49	165	70	88
12.0	a	183	3	92	95	3	3
12.0	b	183	18	121	139	13	4
12.0	c	183	4	145	149	3	2
12.0	d	183	8	103	111	7	1
12.0	e	183	5	146	151	3	5
17.2	a	183	0	135	135	0	0
17.2	b	183	0	155	155	0	0
17.2	c	183	0	141	141	0	0
17.2	d	183	0	169	169	0	0
17.2	e	183	0	155	155	0	0
24.0	a	183	0	-	0	0	0
24.0	b	183	0	-	0	0	0
24.0	c	183	0	-	0	0	0
24.0	d	183	0	-	0	0	0
24.0	e	183	0	-	0	0	0
35.0	a	183	0	-	0	0	0
35.0	b	183	0	-	0	0	0
35.0	c	183	0	-	0	0	0
35.0	d	183	0	-	0	0	0
35.0	e	183	0	-	0	0	0

^ indicates replicate likely not inoculated with proper number of embryos and was omitted from calcula

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: M4

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	115	7	122	94	94
0	b	183	89	3	92	97	97
0	c	183	120	7	127	94	94
0	d	183	111	13	124	90	90
0	e	183	101	4	105	96	96
2.9	a	183	115	7	122	94	94
2.9	b	183	119	13	132	90	90
2.9	c	183	125	11	136	92	92
2.9	d	183	106	14	120	88	88
2.9	e	183	110	8	118	93	93
4.1	a	183	114	18	132	86	86
4.1	b	183	107	16	123	87	87
4.1	c	183	121	9	130	93	93
4.1	d	183	108	14	122	89	89
4.1	e	183	96	11	107	90	90
5.9	a	183	87	19	106	82	82
5.9	b	183	116	9	125	93	93
5.9	c	183	100	19	119	84	84
5.9	d	183	102	16	118	86	86
5.9	e	183	110	17	127	87	87
8.4	a	183	87	16	103	84	84
8.4	b	183	101	13	114	89	89
8.4	c	183	97	10	107	91	91
8.4	d	183	95	19	114	83	83
8.4	e	183	87	17	104	84	84
12.0	a	183	19	10	29	66	66
12.0	b	183	22	7	29	76	76
12.0	c	183	31	17	48	65	65
12.0	d	183	35	14	49	71	71
12.0	e	183	49	17	66	74	74
17.2	a	183	63	16	79	80	80
17.2	b	183	72	27	99	73	73
17.2	c	183	87	24	111	78	78
17.2	d	183	69	40	109	63	63
17.2	e	183	58	23	81	72	72
24.0	a	183	48	44	92	52	52
24.0	b	183	48	38	86	56	56
24.0	c	183	45	30	75	60	60
24.0	d	183	40	26	66	61	61
24.0	e	183	38	30	68	56	56
35.0	a	183	9	119	128	7	7
35.0	b	183	11	120	131	8	8
35.0	c	183	19	129	148	13	13
35.0	d	183	23	62	85	27	27
35.0	e	183	11	105	116	9	9
50.0	a	183	0	-	0	0	0
50.0	b	183	0	-	0	0	0
50.0	c	183	0	-	0	0	0
50.0	d	183	0	-	0	0	0
50.0	e	183	0	-	0	0	0

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: P3

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	133	4	137	97	73
0	b	183	173	12	185	94	95
0	c	183	153	6	159	96	84
0	d	183	152	13	165	92	83
0	e	183	171	8	179	96	93
2.9	a	183	181	8	189	96	99
2.9	b	183	175	4	179	98	96
2.9	c	183	146	7	153	95	80
2.9	d	183	156	6	162	96	85
2.9	e	183	175	6	181	97	96
4.1	a	183	134	6	140	96	73
4.1	b	183	165	12	177	93	90
4.1	c	183	138	19	157	88	75
4.1	d	183	139	17	156	89	76
4.1	e	183	130	12	142	92	71
5.9	a	183	107	20	127	84	58
5.9	b	183	163	20	183	89	89
5.9	c	183	94	7	101	93	51
5.9	d	183	87	16	103	84	48
5.9	e	183	45	9	54	83	25
8.4	a	183	24	163	187	13	13
8.4	b	183	56	143	199	28	31
8.4	c	183	51	125	176	29	28
8.4	d	183	32	99	131	24	17
8.4	e	183	38	124	162	23	21
12.0	a	183	1	158	159	1	1
12.0	b	183	0	-	0	0	0
12.0	c	183	1	128	129	1	1
12.0	d	183	2	129	131	2	1
12.0	e	183	2	118	120	2	1
17.2	a	183	0	-	0	0	0
17.2	b	183	0	-	0	0	0
17.2	c	183	0	-	0	0	0
17.2	d	183	0	-	0	0	0
17.2	e	183	0	-	0	0	0
24.0	a	183	0	-	0	0	0
24.0	b	183	0	-	0	0	0
24.0	c	183	0	-	0	0	0
24.0	d	183	0	-	0	0	0
24.0	e	183	0	-	0	0	0
35.0	a	183	0	-	0	0	0
35.0	b	183	0	-	0	0	0
35.0	c	183	0	-	0	0	0
35.0	d	183	0	-	0	0	0
35.0	e	183	0	-	0	0	0

Sampling Event #3: 9/27/05
Test Initiation Date: 9/28/05
Sample ID: M6

Nominal [Cu] (µg/l)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total Counted	Proportion Normal (%)	Normal Survival (%)
0	a	183	167	3	170	98	91
0	b	183	145	3	148	98	79
0	c	183	135	7	142	95	74
0	d	183	152	2	154	99	83
0	e	183	143	4	147	97	78
2.9	a	183	145	6	151	96	79
2.9	b	183	107	11	118	91	58
2.9	c	183	122	9	131	93	67
2.9	d	183	109	16	125	87	60
2.9	e	183	130	5	135	96	71
4.1	a	183	141	26	167	84	77
4.1	b	183	145	18	163	89	79
4.1	c	183	116	15	131	89	63
4.1	d	183	130	13	143	91	71
4.1	e	183	125	30	155	81	68
5.9	a	183	112	37	149	75	61
5.9	b	183	251	30	281	89	137
5.9	c	183	106	8	114	93	58
5.9	d	183	112	26	138	81	61
5.9	e	183	128	23	151	85	70
8.4	a	183	65	26	91	71	36
8.4	b	183	76	40	116	66	42
8.4	c	183	114	21	135	84	62
8.4	d	183	70	36	106	66	38
8.4	e	183	116	36	152	76	63
12.0	a	183	56	57	113	50	31
12.0	b	183	77	32	109	71	42
12.0	c	183	94	43	137	69	51
12.0	d	183	108	48	156	69	59
12.0	e	183	94	46	140	67	51
17.2	a	183	116	42	158	73	63
17.2	b	183	121	40	161	75	66
17.2	c	183	76	74	150	51	42
17.2	d	183	84	36	120	70	46
17.2	e	183	100	39	139	72	55
24.0	a	183	8	170	178	4	4
24.0	b	183	3	140	143	2	2
24.0	c	183	15	135	150	10	8
24.0	d	183	13	128	141	9	7
24.0	e	183	15	146	161	9	8
35.0	a	183	0	-	0	0	0
35.0	b	183	0	-	0	0	0
35.0	c	183	0	-	0	0	0
35.0	d	183	0	-	0	0	0
35.0	e	183	0	-	0	0	0

APPENDIX E: COPPER MEASURESUREMENTS IN WER TEST SOLUTIONS

Sampling Event #1: 3/31/2004
Test Initiation Date: 4/1/2004

Site	Nominal [Cu] (µg/L)	Total Recov. [Cu] (µg/L)	Dissolved [Cu] (µg/L)
LW 0.0		1.0	0.6
	4.1	5.7	3.0
	5.8	7.6	5.9
	8.4	9.4	6.9
	12.0	12.0	9.4
	17.2	17.4	13.2
M3.1	0.0	1.2	0.7
	4.1	4.6	3.2
	5.8	6.8	4.6
	8.4	9.0	5.6
	12.0	11.2	9.2
	17.2	17.2	13.3
	25	24.4	17.6
	50	53.2	37.7
M3.3	0.0	1.4	1.0
	4.1	5.5	4.1
	5.8	7.3	5.4
	8.4	9.1	6.6
	12.0	12.7	9.7
	17.2	19.5	12.1
	25	25.2	17.3
	50	56.6	29.1
P3	0.0	2.8	1.5
	4.1	6.1	3.9
	5.8	7.5	5.3
	8.4	10.1	7.5
	12.0	12.6	9.1
	17.2	19.3	13.1
	25	26.2	18.5
	50	53.9	39.8
Ref Tox	0.0	1.9	1.1
	4.1	6.1	3.6
	5.8	7.8	5.3
	8.4	9.7	6.7
	12.0	11.4	9.6
	17.2	17.9	12.8

Sampling Event #2: 2/9/2005
Test Initiation Date: 2/11/2005

Site	Nominal [Cu] (µg/L)	Total Recov. [Cu] (µg/L)	Dissolved [Cu] (µg/L)
LW	0	2.6	1.8
	2.9	5.8	3.4
	4.1	6.6	5.4
	5.9	10.0	7.2
	8.4	12.0	7.6
	12	18.2	9.8
	17.2	22.1	14.9
M3.1	0	1.7	1.0
	2.9	4.7	2.9
	4.1	5.8	4.4
	5.9	8.4	6.1
	8.4	12.6	8.1
	12	15.4	11.6
	17.2	20.6	16.6
M4	0	1.4	1.2
	2.9	4.8	3.4
	4.1	5.4	5.6
	5.9	8.7	6.2
	8.4	12.0	8.3
	12	15.1	11.7
	17.2	21.0	15.0
M6	0	1.2	0.7
	2.9	4.1	5.9
	4.1	5.6	5.3
	5.9	9.6	7.7
	8.4	11.6	9.0
	12	16.5	15.0
	17.2	24.2	N/A
P3	0	2.3	1.8
	2.9	5.3	5.1
	4.1	6.3	7.7
	5.9	9.3	6.9
	8.4	13.4	9.4
	12	18.1	12.4
	17.2	20.7	16.3
SN12	0	2.7	1.6
	2.9	5.9	3.4
	4.1	8.0	5.8
	5.9	12.1	7.2
	8.4	13.3	8.4
	12	16.0	11.2
	17.2	23.6	15.0

N/A indicates sample not measured because not necessary for EC50 determination

Sampling Event #3: 9/27/2005
Test Initiation Date: 9/28/2005

Site	Nominal [Cu] (µg/L)	Total Recov. [Cu] (µg/L)	Dissolved [Cu] (µg/L)
LW	0	1.40	1.40
	2.9	N/A	N/A
	4.1	7.04	7.14
	5.9	8.38	9.48
	8.4	11.5	9.7
	12	12.7	12.7
	17.2	19.3	17.7
	24	27.6	25.1
M3.1	0	0.93	0.64
	2.9	N/A	N/A
	4.1	7.46	6.93
	5.9	8.11	7.38
	8.4	11.0	10.4
	12	15.2	11.9
	17.2	20.3	16.3
	24	26.8	23.7
	35	31.4	25.1
M4	0	1.02	0.20
	2.9	N/A	N/A
	4.1	8.27	6.52
	5.9	8.10	6.62
	8.4	12.4	10.1
	17.2	20.8	16.2
	24	28.6	19.2
	35	38.1	29.8
	50	58.3	41.1
M6	0	0.47	0.36
	2.9	N/A	N/A
	4.1	7.19	7.70
	5.9	9.49	7.98
	8.4	11.6	9.44
	12	13.8	10.5
	17.2	19.9	13.9
	24	27.7	19.9
	35	39.7	23.9
P3	0	2.76	2.08
	2.9	N/A	N/A
	4.1	9.89	8.41
	5.9	10.4	9.23
	8.4	13.7	11.4
	12	16.6	12.5
	17.2	22.4	16.8
	24	30.6	24.5
	35	41.2	33.9
SN12	0	0.86	0.65
	2.9	N/A	N/A
	4.1	7.51	6.65
	5.9	8.85	7.03
	8.4	13.1	10.7
	12	14.6	10.6
	17.2	20.6	14.5
	24	27.8	19.4
	35	40.0	28.2

N/A indicates sample not measured because not necessary for EC50 determination

APPENDIX F: COPPER TOXICITY EVALUATION OF AMBIENT WATER FROM MUKILTEO: SAMPLE COORDINATION WITH NORTHWEST FISHERIES SCIENCE CENTER'S (NWFSC) INVESTIGATION OF CU EFFECTS ON CHINOOK SALMON SMOLTS IN SALT WATER AT THE MUKILTEO FIELD STATION

Copper Toxicity Evaluation of Ambient Water from Mukilteo: Sample Coordination with Northwest Fisheries Science Center's (NWFSC) Investigation of Cu Effects on Chinook Salmon Smolts in Salt Water at the Mukilteo Field Station

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ABSTRACT

The results of chemistry and toxicity analysis are reported for seawater samples conducted in September 2008 from the Mukilteo Field Station, Washington. The samples were analyzed to provide additional data on saltwater chemistry and toxic effects of copper (Cu) to mussel (*Mytilus galloprovincialis*) embryos in the same source water used to assess the effects of Cu exposure to sublethal olfactory impairment in Chinook salmon (*Onchorhynchus tshawytscha*) smolts (David Baldwin, NWFSC, personal communication). The average dissolved and total Cu measured in samples collected from the seawater flow-through system of the Mukilteo Field Station was 0.15 µg/L (stdev 0.03, range, 0.1 to 0.19µ/L) and 0.18 µg/L (stdev 0.01, range 0.16 to 0.2 µg/L), respectively. The filtered Cu concentration (dissolved) accounted for about 87% of the total Cu present. Total and dissolved organic matter averaged about 1.5 mg/L, suggesting that the organic carbon was present mainly in the dissolved phase. The samples also had relatively low concentrations of suspended solids averaged 13 mg/L (range, 6 to 30 mg/L). The normal Survival EC50s for mussel embryos calculated from the measured dissolved Cu concentrations ranged from 5.2 to 5.87 µ/L and the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) calculated for the Mukilteo Field Station samples were 4.1 and 5.8 µg/L, respectively. The EC50s calculated for the seawater from the Mukilteo Field Station were much lower than the EC50s determined for samples of ambient water (nearshore and marine) from Sinclair and Dyes Inlets, and were more than a factor of two below the regression reported by Arnold et al. (2006; $EC50 = 11.2DOC^{0.6}$), derived from WER studies conducted throughout North America. Seawater from the Mukilteo Field Station had very little binding capacity for Cu, and consequently, mussel embryos were very sensitive to Cu exposure.

INTRODUCTION

This document describes the results of sample collection and analysis conducted in collaboration with National Oceanic and Atmospheric Administration (NOAA) Fisheries, Northwest Fisheries Science Center's (NWFSC) investigation of copper (Cu) effects on Chinook salmon (*Onchorhynchus tshawytscha*) smolts in saltwater from Puget Sound conducted in September 2008 at NWFSC's Mukilteo Field Station (Figure F-1, David Baldwin, NWFSC, Seattle, WA, personal communication). The samples were analyzed to provide additional data on saltwater chemistry and toxic effects to mussel (*Mytilus galloprovincialis*) embryos in the same source water used for the Chinook smolt study.

BACKGROUND

As part of the cooperative ENVironmental inVESTment (ENVVEST) project being conducted by the Puget Sound Naval Shipyard & Intermediate Maintenance Facility (PSNS&IMF), U.S. Environmental Protection Agency (U.S. EPA), Washington Department of Ecology (Ecology) and local stakeholders, the Space and Naval Warfare Systems Center, Pacific (SSC Pacific), and other participants have been addressing water quality issues in the Sinclair/Dyes Inlet watershed in the Puget Sound (ENVVEST 2006). The ENVVEST working group has conducted monitoring and modeling studies in Sinclair and Dyes Inlets including assessing contaminant loading from the watershed (Brandenberger et al., 2007, Cullinan et al., 2007), evaluating the toxicity of Cu in ambient waters to mussel embryos (Rosen et al., 2006), and assessing the bioaccumulation and ecological effects of Cu and other contaminants on demersal fish and invertebrates (Johnston et al., 2007).

Figure F-1. Location of Mukilteo Field Station and Sinclair Inlet in the Central Puget Sound, WA.

Recently, studies conducted at NWFSC reported that juvenile salmonids were sensitive to Cu exposure in freshwater (sublethal olfactory impairment, Hecht et al., 2007) and that "water chemistry

parameters in freshwater were less protective at the fish nose than at the fish gill against toxicity from dissolved copper" (McIntyre, Baldwin, Meador, and Sholtz, 2008). However, the abundance and composition of ligands and organic matter (which bind to and detoxify metals) is different in salt water than in fresh water and it is unclear how Cu would effect older life stages of salmonids that have acclimatized to saltwater (McIntyre et al. 2008). Recently, investigations were conducted by NWFSC to evaluate the effect of Cu exposure on olfactory impairment on Chinook smolts in saltwater (David Baldwin, NWFSC, Seattle, WA, personal communication). These experiments were conducted at NWFSC's Mukeltio Field Station using the flow-through seawater system to deliver site water from the Puget Sound for the experimental manipulations (dosing with Cu).

TECHNICAL APPROACH

Samples of the site water collected directly from the Mukilteo Field Station flow-through system used for the Chinook smolt study were analyzed for seawater chemistry and toxicity to mussel embryos. Samples were collected for chemistry analysis on September 11, 15, 18, and 23, 2008, during the Chinook smolt study. On September 18, 2008, triplicate 1-L samples were collected and sent overnight to SSC Pacific for the mussel embryo toxicity testing. The purpose of the study was to establish the sensitivity of Cu exposure to mussel embryos in site water from the Mukilteo Field Station for comparison to smolt sensitivity to Cu exposure. Details of the sampling and results are described below.

METHODS

TOXICITY TESTS

Toxicity testing was conducted with embryos of the Mediterranean mussel (*Mytilus galloprovincialis*). This species and life stage is relevant because embryogenesis is impacted by copper at very low concentrations (e.g., < 10 ppb; U.S. EPA, 1995b), and the species is commercially important in the Puget Sound area (Taylor Shellfish Farms, 2004). The 48-hour embryo-larval development endpoint for *Mytilus* sp. is the driver of the current saltwater ambient Water Quality Criteria (WQC) of 4.8 (acute) and 3.1 (chronic) µg dissolved Cu/L (U.S. EPA 1995b, State of Washington, 2006), and is recommended by the EPA for use in Water Effect Ratio (WER) studies (U.S. EPA 1994a, 2001). The *M. galloprovincialis* embryos used in this study were obtained from Carlsbad Aquafarm, Carlsbad, CA (http://www.carlsbadaquafarm.com/).

The toxicity tests were conducted at the SSC Pacific Bioassay Laboratory (SSC Pacific, 2009), which is accredited by the State of California Department of Health Services and the State of Washington Department of Ecology for a number of whole effluent toxicity (WET) testing methods, through the Environmental Laboratory Accreditation Program (ELAP). Toxicity tests were conducted following American Society for Testing and Materials (ASTM) and U.S. EPA guidance for whole effluent toxicity (ASTM, 1999; U.S. EPA, 1995a) and for determining WERs (U.S. EPA, 1994a). Briefly, site and laboratory water samples were spiked with as many as eight nominal copper concentrations, including 0 (control), 2.9, 4.1, 5.9, 8.4, 12.0, 17.2, 24.0, and 50.0 µg/L. Laboratory water used for the study consisted of filtered (0.45 µm), open coastal seawater collected from the research pier at Scripps Institute of Oceanography (SIO), San Diego, California and from the University of California Davis Marine Pollution Studies Laboratory at Granite Canyon (GC), Carmel, California (http://www.envtox.ucdavis.edu/ GraniteCanyon/). An additional laboratory control sample with high Dissolved Organic Carbon (DOC) (20 mg/L) was made in clean seawater (filtered 0.45 um, from SSC Pacific Cold Room) using Suwannee River freeze-dried dissolved organic carbon (SWN DOC).

Copper stock solutions were made from copper sulfate and confirmed by stabilized temperature graphite furnace atomic absorption (STGFAA) spectroscopy prior to use. The same stock solution was used for laboratory waters, site waters, and any associated reference toxicant tests. To account for any change in Cu concentration compared to initial concentrations, a sixth replicate for at least one test concentration per sample was also included in the test for quantification of dissolved copper at the end of the test.

An equilibration period of at least 3 to 5 hours following copper additions was allowed prior to addition of embryos. After 48 hours of exposure, normally developing mussel embryos should have achieved the prodissoconch I stage, which is characterized by a straight-hinged, D-shaped larval shell. Two different endpoints were used to assess larval development: percent of surviving larvae that developed normally (Normal Survival) and the proportion of surviving larvae that were normal (Proportion Normal). The data were used to obtain the No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), and effects concentration (ECx) from the site water and the lab water, where x ranged from 1, 5, 10, 15, … 95, 99% effect levels. The WER is the ratio between the EC50 obtained for the site water and the EC50 from the lab water, which provides a measure of the detoxifying capacity of the site water. The EC50 from the site water for mussel embryos can also be compared to the inhibition concentration (IC50) for sublethal olfactory impairment obtained from Cu toxicity testing using Chinook smolts (David Baldwin, NWFSC, personal communication).

The toxicity tests were conducted in accordance with the SSC Pacific Bioassay Laboratory Quality Assurance (QA) Plan for all aspects of testing, including the source, handling, condition, receipt, and proper storage of samples and test organisms, as well as the appropriate calibration and maintenance of instruments and equipment. All data generated by the laboratory were evaluated for completeness and accuracy. Appropriate laboratory controls were conducted with each test, and were required to meet specific test acceptability criteria. For the mussel test, greater than or equal to 70% Normal Survival in the controls is required for the test to be acceptable. In addition, reference toxicant tests were conducted with each test as a measure of the laboratory's performance and test batch sensitivity. Reference toxicant EC50 values are must be within two standard deviations of the running mean.

SEAWATER CHEMISTRY

Samples of site water were analyzed for total and dissolved Cu, DOC, total organic carbon (TOC), total suspended solids (TSS), and salinity. Sampling protocols for the site waters followed EPA Method 1669, EPA's Trace Metals Sampling Technique (U.S. EPA, 1995c). These include the use of acid-cleaned materials made of polyethylene, and "clean hands/dirty hands" techniques. SSC Pacific provided pre-cleaned, acid-washed sample bottles and a filtering apparatus for sample collection by Mukilteo Field Station personnel. Preservation, handling, and analysis of the samples were conducted in class-100 trace metal clean working areas and quartz-still grade nitric acid (Q-HNO_3) was used for sample preparation.

Copper concentrations were measured with a Perkin-Elmer SCIEX ELAN DRCII Inductively Coupled Plasma Mass Spectrometer (ICPMS) with a Dynamic Reaction Cell (DRC) and an in-line preconcentration Flow Injection Analysis System (FIAS) 400. This setup is specifically configured for trace metal analysis, with the FIAS performing an in-line sample treatment and conditioning, and a secondary in-line reaction chamber with ammonia gas to eliminate interferences and lower detection limits. Undiluted samples and standards were injected directly into the FIAS via a Perkin-

Elmer Autosampler 100. Analytical standards were made with Perkin-Elmer multi-element standard solution (PEMES-3) diluted in seawater acidified with reagent grade nitric acid (Q-HNO_3), and were analyzed at the beginning and end of the run. The analysis also included measurement of analytical blanks made up of 1N Q-HNO_3 after every five samples. The detection limit for Cu in seawater was 0.057 µg/L (parts-per-billion, or ppb); the mean and standard deviation percent recovery on spiked samples was 102% ±9%, and the relative percent recovery on duplicate samples was 99 to 101%.

Copper complexation capacity (CuCC) is a chemical measurement defined as the capacity of ambient water to assimilate inputs of Cu without associated adverse effects upon aquatic organisms. It is measured with a Cu ion selective electrode (CuISE), in response to systematic addition of Cu in ambient water. The response of the CuISE is indicative of the concentration of aqueous free Cu ion ($Cu(II)_{aq}$) in solution, which according to the free-ion model (Buffle, Altman, Fillela, and Tessier, 1990), and substantiated by experimental evidence (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Ericksen, Mackey, Vanbam, and Nowak, 2001; Rivera-Duarte and Zirino 2004), is the fraction of Cu that is available to organisms, making it a better predictor of potential Cu toxicity than either the total or dissolved Cu concentrations. Note CuCC samples were not analyzed as part of this study.

TOC, DOC, and TSS samples were analyzed by CAS, Kelso, Washington. The organic carbon samples were analyzed according to EPA Method 415.1, with a target reporting limit of 0.5 mg/L. The TSS samples were analyzed according to EPA Method 160.2, with a target reporting limit of 0.5 mg/L. Salinity was measured with a salinity meter with an accuracy of 0.01 psu and a resolution of 0.001 psu.

Site water samples were collected from the Mukilteo Field Station flow-through system at a location that was representative of the water used in the Chinook smolt study and free from any obvious source of contamination. Pre-cleaned 1-L, 500-ml, and 125-ml high-density polyethylene (HDPE) sample bottles were used for collecting Cu or toxicity samples using clean techniques (U.S. EPA, 1995c) by filling the bottles with flow-through water from the laboratory system after rinsing the sample bottle three times with the sample water before capping. The weekly sampling consisted of collecting two 125-ml sample bottles of whole water, two 125-ml sample bottles of seawater filtered with pre-cleaned (0.45 um) filter assembly, and one 1-L polycarbonate sample bottle for TSS. The one-time sampling for toxicity testing consisted of collecting three replicate 1-L HDPE bottles filled full, following the technique described above, stored on ice and shipped overnight to SSC Pacific for processing. A summary of the samples collected is provided in Table F-1.

During the first sampling event on September 11, 2008, an additional 500-ml HDPE sample bottle was filled with E-pure™ water supplied by SSC Pacific. In addition a 125-ml filtration sample was collected using the E-pure™ water. These samples were used as field blanks for the study.

DATA ANALYSIS

The normal survival data were used to calculate EC50s with ToxCalc™ version 5.0, using the Maximum Likelihood Probit method. The EC50 and WER values were calculated from nominal and dissolved copper concentrations for each test. WERs for each site water sample were calculated by dividing the site water EC50 by the associated lab water (GC) EC50. NOEC and LOEC were obtained from hypothesis testing following arc-sine square root transformations of the toxicity data, and verification of normal distribution of data and homogeneity of variances using Shapiro-Wilkes and Bartlett's tests, respectively. See Table F-1.

Table F-1. Summary of samples collected.

A. Sample Description

Weekly Sampling	Bottle	Target Reporting Limit	Target Detection Limit
Total Cu	125-ml HDPE	0.19 µg/L	0.057 µg/L
Dissolved Cu	125-ml HDPE	0.19 µg/L	0.057 µg/L
TOC	125-ml HDPE w/acid	0.5 mg/L	
DOC	125-ml HDPE w/acid	0.5 mg/L	
TSS	one 1-L poly	0.5 mg/L	
CuCC	four 500-ml HDPE bottles pre-filled with DI water	50 nM	25 nM
One Time Sampling			
Toxicity	three 1-L HDPE		

B. Sampling Event

	Samples Collected					
	Total Cu	Diss. Cu	CuCC[1]	DOC/TOC	TSS	Toxicity[2]
9/11/2008	2	2	4	2	1	
9/11/2008 Blank	1	1				
9/15/2008	2	2	4	2	1	3
9/18/2008	2	2	4	2	1	
9/23/2008	2	2	4	2	1	
Split Sample[3]						
Total Samples	9	9	16	8	4	3

[1] CuCC samples were not analyzed as part of this study.
[2] Toxicity samples were also analyzed for dissolved Cu
[3] Split samples were analyzed at the discretion of the Mukilteo Field Lab

RESULTS AND DISCUSSION

SEAWATER CHEMISTRY

The results obtained for total and dissolved Cu, DOC, TOC, and TSS for the weekly samples and the dissolved Cu measured in the toxicity samples are summarized in Table F-2. The field blank sample, which consisted of E-Pure™ water provided by SSC Pacific and used to fill sample bottles during the September 11, 2008 sampling event was elevated for total and dissolved Cu. However, since all the other samples collected had much lower Cu concentrations, it is unlikely that whatever caused the elevated levels in the field blank affected any of the other samples. The average dissolved Cu measured in samples collected from the seawater flow-through system of the Mukilteo Field Station was 0.15 µg/L (stdev, 0.03; range, 0.1 to 0.19 µg/L) and the average total Cu was 0.18 µg/L (stdev, 0.01; range, 0.16 to 0.2 µg/L). The filtered Cu concentration (dissolved) accounted for about 87% of the total Cu present. The close agreement between DOC (1.5 mg/L; range, 1.4 to 1.9 mg/L) and TOC (1.5 mg/L; range, 1.1 to 1.6 mg/L) suggests that organic carbon was present mainly in the dissolved form. The samples also had relatively low concentrations of suspended solids averaging 13 mg/L and ranging from 6 to 30 mg/L. The dissolved Cu measured in the toxicity samples collected on September 15, 2008 were about twice as high as the weekly samples collected on the same day; however, the toxicity samples had similar dissolved Cu concentrations (see Table F-2).

Table F-2. Summary of analytical chemistry results for the weekly and toxicity samples from the Mukilteo Field Station.

Weekly Samples	**Weekly Samples**	**Cu µg/L**			**mg/L**		
Date	Sample ID	Dissolved	Total	%Diss	DOC	TOC	TSS
9/11/2008	CuBlank[1]	0.548	0.588				
9/11/2008	Cu1	0.163	0.181	90%	1.4	1.6	7
	Cu2	0.178	0.203	88%	1.5	1.6	
9/15/2008	Cu1	0.160	0.174	92%	1.4	1.4	9
	Cu2	0.175	0.161	109%	1.5	1.1	
9/18/2008	Cu1	0.114	0.173	66%	1.5	1.6	30
	Cu2	0.148	0.182	82%	1.4	1.5	
9/23/2008	Cu1	0.094	0.166	57%	1.4	1.6	6
	Cu2	0.188	0.168	112%	1.9	1.2	
Average of Weekly Samples		0.153	0.176	87%	1.5	1.5	13
Toxicity Samples							
9/15/2008	NOAA1	0.399					
9/15/2008	NOAA2	0.311					
9/15/2008	NOAA3	0.416					
Average of Toxicity Samples		0.375					

[1] E-Pure™ Water provided by SSC Pacific

TOXICITY RESULTS

The raw data from the toxicity tests for water quality conditions during the test, number of normal and abnormal individuals, percent normal, percent survival, and statistical significance for the Cu exposures tested in waters from SIO, GC, NOAA1, NOAA2, NOAA3, and SWN DOC are reported in Attachment 2. The SIO water resulted in a failed test due to low normal survival (< 70%) in the controls (no Cu added treatment). Therefore, all comparisons were made with the GC lab water. Other than the SIO sample, all the other tests resulted in successful toxicity tests, with no deviations from targeted test conditions and test acceptability criteria. All reference toxicant tests resulted in EC50 values that were within two standard deviations of the testing laboratory's running mean, according to current control charts at the SSC Pacific Bioassay Laboratory (data not shown).

The tests were initiated within the 96-h holding time requirement for WER studies (U.S. EPA, 2001). The test pH, temperature, salinity, and dissolved oxygen concentrations were within targeted ranges for all measurements (Attachment 2). The measured dissolved Cu concentrations were similar to the nominal concentrations and showed good agreement with the Cu doses required for the experiment (Table F-3). The relatively high toxicity in the SIO controls (no Cu added treatment) was probably due to elevated Cu (5.3 µg/L) measured in ambient water collected from Scripps Pier (Table F-3).

Table F-3. The nominal and measured dissolved Cu concentrations in each of the experimental treatments.

	Dissolved Concentration (ug/L)					
Nominal (µg/L)	Lab SIO	Lab GC	NOAA1	NOAA2	NOAA3	SWN DOC
0	5.27	0.357	0.399	0.311	0.416	NM
2.9	7.99	2.37	2.68	2.36	2.58	NM
4.1	9.14	3.53	3.77	3.9	3.52	6.54
5.8	10.54	6.73	5.65	5.19	5.08	9.91
8.4	14.73	9.03	7.37	7.55	11.45	9.53
12	20.25	12.66	17.6	11.52	13.98	12.16
17.2	NM	NM	NM	NM	NM	20.46
24	NM	NM	NM	NM	NM	25.95

NM= Not Measured (not needed for calculations)

A strong dose-response relationship was observed for each of the Mukilteo Field Station samples (NOAA1, NOAA2, NOAA3) and for the reference samples from Granite Canyon (GC) and Suwannee River DOC (SWN DOC) (Figure F-2, see Attachment 3 for results of statistical analysis). The Normal Survival EC50s calculated from the measured dissolved Cu concentrations in the Mukilteo Field Lab samples ranged from 5.2 to 5.87 µg/L and were slightly lower than the EC50s based on the nominal Cu concentrations (Table F-4).

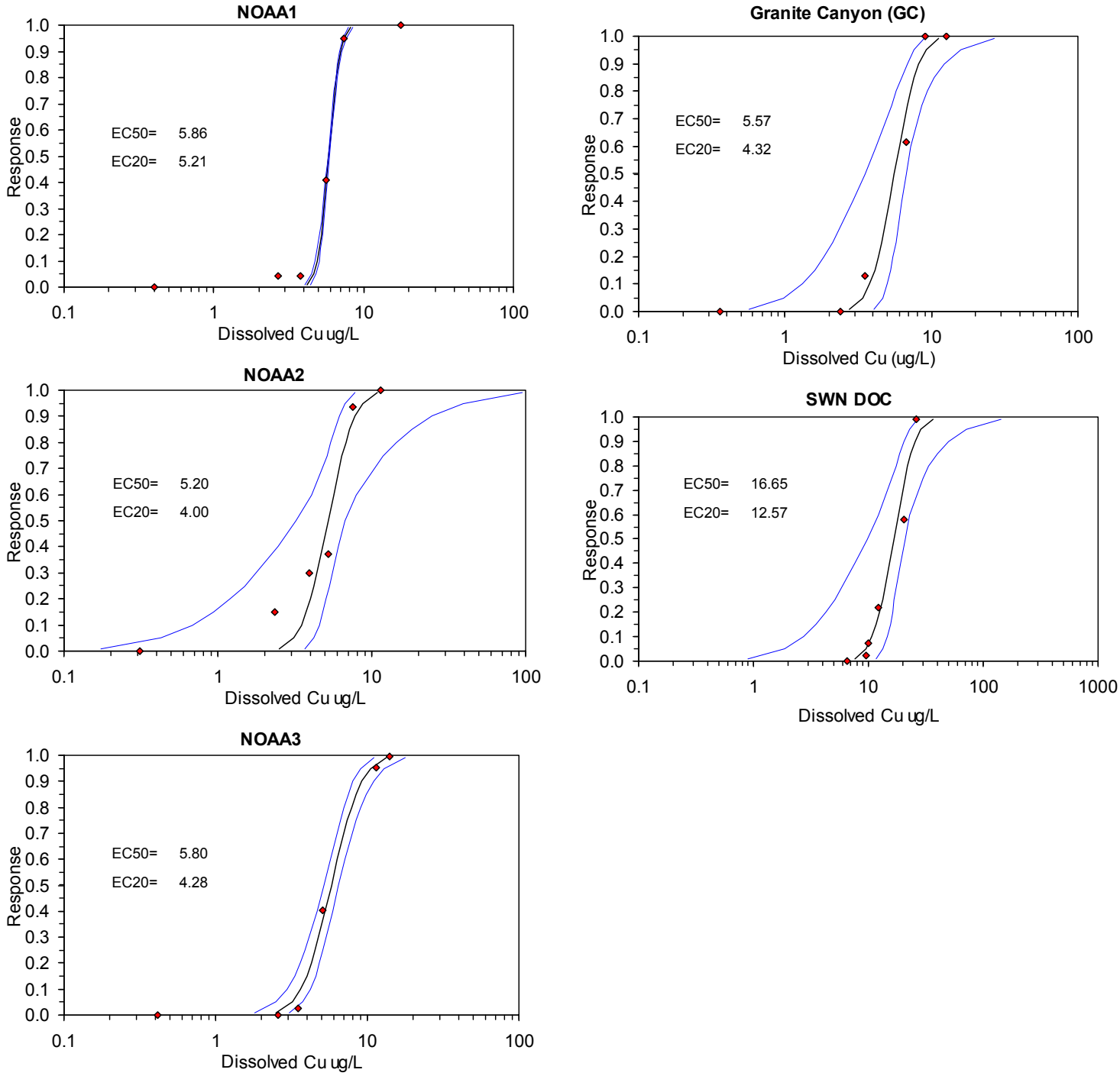

Figure F-2. The dose-response curves calculated for measured dissolved Cu exposure to mussel embryos in samples of seawater from NOAA1, NOAA2, NOAA3, GC, and SWN DOC.

Table F-4. Results from statistical analysis of toxicity data for Normal Survival based on nominal and measured dissolved Cu concentrations.

	Normal Survival Endpoint - Nominal Cu Data						
Sample ID	NOEC (µg/L)	LOEC (µg/L)	EC50 (µg/L)	95% C.L. (µg/L)	% Normal Survival in Controls[b]	Standard Deviation	**Nominal WER**
GC	2.9	4.1	5.30	5.1-3.5	93.5	9.9	
SIO	N/A	N/A	N/A	N/A	50.2	7.3	
SWN DOC	12.0	17.2	15.52	13.3-17.2	83.6	6.2	**2.93**
NOAA1	4.1	5.8	6.11	6.0-6.2	89.6	10.3	**1.15**
NOAA2	4.1	5.8	5.84	4.6-6.8	99.3	8.5	**1.10**
NOAA3	4.1	5.8	6.06	5.8-6.3	91.9	10.0	**1.14**
NOAAavg			**6.00**				**1.13**

	Normal Survival Endpoint - Measured Dissolved Cu Data				
Sample ID	NOEC (µg/L)	LOEC (µg/L)	EC50 (µg/L)	95% C.L. (µg/L)	**Dissolved WER**
GC	2.9	4.1	**5.57**	3.5 - 6.72	
SIO	N/A	N/A	N/A	N/A	
SWN DOC	12.0	17.2	16.65	9.7 - 20.73	**2.99**
NOAA1	4.1	5.8	5.87	5.8 - 5.95	**1.05**
NOAA2	4.1	5.8	5.20	3.2 - 6.75	**0.93**
NOAA3	4.1	5.8	5.80	5.1 - 6.48	**1.04**
NOAAavg			**5.61**		**1.01**

[b] Normal survival values >100% are reported as 100% in Toxcalc 5.0 analysis

The NOECs and LOECs calculated for the Mukilteo Field Station, GC, and SWN DOC samples were 4.1 and 5.8 µg/L, 2.9 and 4.1 µg/L, and 12.0 and 17.2 µg/L dissolved Cu, respectively (Table F-4). The NOEC and LOEC for seawater samples from the Mukilteo Field Station were above the WQS for dissolved Cu of 3.1 µg/L for chronic and 4.8 µg/L for acute exposure (State of Washington, 2006). The lower bound of the 95th percentile of the EC50s for GC and NOAA2 were close to the chronic limit, while lower bounds for NOAA1 and SWN DOC were greater than or equal to the acute limit. The WERs calculated for Mukilteo Field Station seawater ranged from 0.93 to1.05 with an average WER of 1.01, indicating that the detoxifying capacity of the seawater from the Mukilteo Field Station was very similar to the lab water from Granite Canyon on the central California coast. The high DOC in the SWN DOC sample resulted in the highest WER in the samples tested (Table F-4).

The relationship between the EC50s calculated for the seawater from the Mukilteo Field Station and Sinclair Inlet as a function of DOC is shown in Figure F-3. The EC50s calculated for the seawater from the Mukilteo Field Station were much lower than the EC50s determined for samples of ambient water (near shore and marine) from Sinclair and Dyes Inlets, and were more than a factor of two below the regression reported by Arnold et al. (2006; $EC50 = 11.2DOC^{0.6}$), derived from WER studies conducted throughout North America. This indicates that there was very little binding capacity for Cu in the seawater sampled from the Mukilteo Field Station. The results also show that mussel embryos were very sensitive to Cu exposure in Mukilteo Field Station seawater.

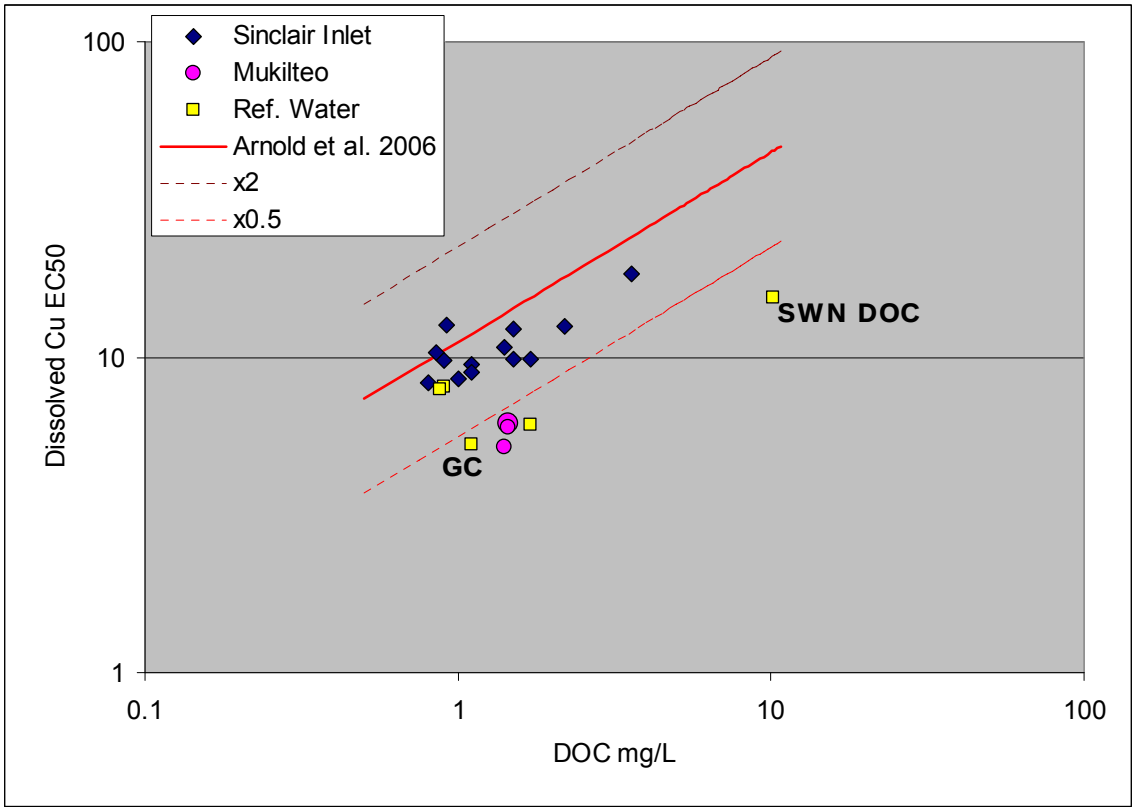

Figure F-3. Comparison of the dissolved Cu EC50 as a function of DOC for seawater samples from the Mukilteo Field Station (circles), Sinclair Inlet (diamonds), and reference stations (squares).

UNCERTAINTY

The blank samples consisted of E-Pure™ water that were sent to the Mukilteo lab. These samples had relatively high ~0.5 ppb levels of Cu compared to the other samples. Additionally, the toxicity samples collected on September 15, 2008 (three 1-L samples) were about twice as high as the weekly sample (125-ml filtered sample) taken on the same day. It is unclear what caused these differences, although variations from sample contamination are typical when working at ultra-trace level concentrations (< 1 ppb). It is unlikely that these inconsistencies affected the outcomes of the tests, because Cu levels measured in the toxicity samples were very similar (0.3 to 0.4 µg/L dissolved Cu, Figure F-3) and the Cu additions used in toxicity tests were much higher than ambient concentrations.

The results from this study showed that the mussel embryos were very sensitive to Cu exposure in site water from the Mukilteo Field Station. However, care should be taken to not over interpret the limited data available from this study. The dissolved Cu fraction in the weekly samples accounted for the majority of Cu present (87%; range 57 to 112%, Figure F-3), and dissolved Cu exceeded total Cu in two samples and DOC was also higher than TOC in the same samples. One issue that might be a problem is the DOC and TOC measurements. The samples were very low in organic matter and very close to the DOC detection limit (ostensibly 0.5 mg/L but more realistically ~1 mg/L). If the DOC levels were actually < 1 mg/L then the observed toxicity would fall within the range reported from other studies (Figure F-3). The organic carbon was analyzed by direct injection (EPA Method 415.1; U.S. EPA, 1974) without any pre-concentration, which probably did not provide the sensitivity and precision needed to accurately determine organic matter concentrations in the samples. Samples with

trace organic carbon levels (≤ 1 mg/L) require the use of more precise oceanographic methods for analyzing trace levels of DOC/TOC in marine waters.

CONCLUSIONS

Samples of seawater obtained from the Mukilteo Field Station were analyzed to provide data on saltwater chemistry and toxic effects of Cu to mussel (*Mytilus galloprovincialis*) embryos in the same source water used for the study of the effects of Cu on sublethal olfactory impairment in Chinook smolts. The average dissolved and total Cu measured in samples collected from the seawater flow-through system of the Mukilteo Field Station was 0.15 µg/L (stdev, 0.03; range, 0.1 to 0.19 µg/L) and 0.18 µg/L (stdev, 0.01; range, 0.16 to 0.20 µg/L), respectively. The filtered Cu concentration (dissolved) accounted for about 87% of the total Cu present. Total and dissolved organic matter averaged about 1.5 mg/L, suggesting that the organic carbon was present mainly in the dissolved phase. The samples also had relatively low concentrations of suspended solids averaging 13 mg/L (6 to 30 mg/L). The mussel embryo Normal Survival EC50s calculated from the measured dissolved Cu concentrations ranged from 5.2 to 5.87 µg/L and the NOECs and LOECs were 4.1 and 5.8 µg/L dissolved Cu, respectively. The NOEC and LOEC for seawater samples from the Mukilteo Field Station were above the water quality standards for dissolved Cu of 3.1 µg/L for chronic and 4.8 µg/L for acute exposure (State of Washington, 2006). Seawater from the Mukilteo Field Station had very little binding capacity for Cu, and consequently, mussel embryos were very sensitive to Cu exposure.

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Attachment 1: Sampling Procedures

NOTE: All sampling procedure conducted following ultra-clean sampling protocols (eg. “clean hands” and “dirty hands”).

Freq	Sample	Bottle	Procedure	Storage
First time	Total Cu blank	125 ml	Rinse three times with lab water (from CuCC bottle), fill with syringe, without filter to neck, cap	Refrigerate
First time	Dissolved Cu blank	125 ml	Place filter on syringe, filter sample into bottle, need at least 60 ml; Waste first 3-5 drops through the filter	Refrigerate
Each time	Total Cu	125 ml	Rinse three times with sample, fill inverted to neck, cap	Refrigerate
Each time	TOC	125 ml w/acid	Fill with syringe, without filter	Refrigerate
Each time	Dissolved Cu	125 ml	Place filter on syringe, filter sample into bottle, need at least 60 ml; waste first 3to5 drops through the filter	Refrigerate
Each time	DOC	125 ml w/acid	Fill with syringe and filter, need at least 60 ml	Refrigerate
Each time	TSS	1 L	Rinse three times, fill with sample water full	Refrigerate
Each time	CuCC	2 to 500 ml	Remove bottles, pour out lab water, rinse three times with sample, fill inverted, then dump out about 25% of sample (¾ full) to leave room for expansion from freezing	Frozen
One time	Cu Toxicity	6 to 1 L	Rinse three times with sample, fill inverted, then cap full	ship to SSC San Diego overnight on ice packs
One time	Collect duplicate total and dissolved samples		Repeat sampling described above	

Attachment 2: Water Quality Parameters for Toxicity Tests of samples from Mukilteo Field Station

Sample ID	Nominal [Cu] (µg/l)	pH (SU)			D.O. (mg/l)			Temperature (°C)			Salinity (‰)		
		Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
SIO	0	8.1	7.9	8.0	7.7	6.5	7.0	15.0	14.2	14.5	29.1	29.0	29.0
	4.1	8.0	8.0	8.0	7.8	6.6	7.1	15.0	14.2	14.5	29.5	29.0	29.2
	8.4	8.0	8.0	8.0	7.8	6.8	7.2	15.0	14.2	14.5	29.6	29.0	29.2
	17.2	8.0	8.0	8.0	7.8	7.0	7.5	15.0	14.2	14.5	29.7	29.2	29.4
	35	8.0	8.0	8.0	7.8	7.0	7.5	15.0	14.2	14.5	29.8	29.2	29.4
GC	0	8.0	7.9	7.9	7.7	6.9	7.3	14.5	14.2	14.4	29.4	29.1	29.3
	4.1	8.0	7.9	7.9	7.7	7.0	7.4	14.5	14.2	14.4	30.3	29.9	30.1
	8.4	8.0	7.9	7.9	7.9	7.0	7.5	14.5	14.2	14.4	30.5	30.0	30.2
	17.2	8.0	7.9	7.9	8.0	7.0	7.5	14.5	14.2	14.4	30.5	29.6	30.0
	35	8.0	7.9	7.9	8.0	7.0	7.5	14.5	14.2	14.4	30.6	30.1	30.3
NOAA 1	0	7.9	7.7	7.9	7.9	7.3	7.5	14.7	14.2	14.4	29.8	29.0	29.4
	4.1	7.9	7.7	7.9	8.1	7.2	7.6	14.7	14.2	14.4	30.5	30.1	30.3
	8.4	7.9	7.8	7.9	8.1	7.0	7.5	14.7	14.2	14.4	30.7	30.2	30.4
	17.2	8.0	7.8	7.9	8.1	7.2	7.6	14.7	14.2	14.4	30.8	30.3	30.5
	35	8.0	7.8	7.9	8.1	7.2	7.6	14.7	14.2	14.4	31.1	30.4	30.7
NOAA 2	0	7.9	7.7	7.8	8.1	7.1	7.5	14.4	14.2	14.3	29.8	29.6	29.7
	4.1	7.9	7.7	7.9	8.0	7.1	7.5	14.4	14.2	14.3	30.6	30.2	30.3
	8.4	7.9	7.8	7.9	8.0	7.3	7.6	14.4	14.2	14.3	30.7	30.2	30.4
	17.2	7.9	7.7	7.9	8.0	7.3	7.5	14.4	14.2	14.3	30.7	30.4	30.5
	35	7.9	7.8	7.9	7.9	7.3	7.5	14.4	14.2	14.3	30.6	30.2	30.3
NOAA 3	0	7.9	7.7	7.8	7.8	6.8	7.3	14.7	14.2	14.4	30.0	29.5	29.8
	4.1	7.9	7.7	7.8	7.9	6.9	7.4	14.7	14.2	14.4	30.6	30.3	30.4
	8.4	7.9	7.7	7.9	7.9	6.9	7.4	14.7	14.2	14.4	30.8	30.3	30.5
	17.2	7.9	7.8	7.9	7.9	7.3	7.5	14.7	14.2	14.4	30.6	30.4	30.5
	35	7.9	7.7	7.9	7.9	7.2	7.5	14.7	14.2	14.4	30.9	29.2	30.2
DOC	0	7.9	7.8	7.9	7.8	6.6	7.2	15.1	14.2	14.6	33.1	32.7	32.8
	12	7.9	7.8	7.9	7.9	6.7	7.2	15.1	14.2	14.6	33.9	33.8	33.8
	50	7.9	7.8	7.9	7.9	6.7	7.2	15.1	14.2	14.6	34.1	33.8	34.0
LAB	0	8.0	7.9	8.0	7.8	6.8	7.2	15.1	14.2	14.6	33.4	32.9	33.1
	4.1	8.0	7.9	8.0	7.9	6.8	7.5	15.1	14.2	14.6	34.1	33.8	34.0
	8.4	8.0	7.9	8.0	7.9	6.8	7.5	15.1	14.2	14.6	34.2	33.8	34.0
	12	8.0	7.9	8.0	8.0	6.8	7.5	15.1	14.2	14.6	34.1	33.8	34.0

Attachment 3: Toxicity Test Data for Samples from Mukilteo Field Station

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
SIO	0	a	151	90	67	157
		b	151	78	67	145
		c	151	71	66	137
		d	151	64	61	125
	2.9	a	151	10	162	172
		b	151	7	134	141
		c	151	6	135	141
		d	151	10	114	124
	4.1	a	151	0	160	160
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	5.9	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	8.4	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	12.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	17.2	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	24.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*

Italics indicates estimate. When no normal larvae were observed, abnormals were estimate

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
GC	0	a	151	142	9	151
		b	151	154	11	165
		c	151	120	38	158
		d	151	149	12	161
	2.9	a	151	134	27	161
		b	151	141	12	153
		c	151	157	19	176
		d	151	173	16	189
	4.1	a	151	138	27	165
		b	151	108	11	119
		c	151	113	15	128
		d	151	130	12	142
	5.9	a	151	59	103	162
		b	151	46	126	172
		c	151	58	86	144
		d	151	52	67	119
	8.4	a	151	0	160	160
		b	151	0	144	144
		c	151	0	152	152
		d	151	0	127	127
	12.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	17.2	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	24.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*

Italics indicates estimate. When no normal larvae were observed, abnormals were estimated.

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
NOAA 1	0	a	151	154	9	163
		b	151	123	13	136
		c	151	122	9	131
		d	151	142	8	150
	2.9	a	151	130	12	142
		b	151	118	10	128
		c	151	145	10	155
		d	151	121	5	126
	4.1	a	151	152	13	165
		b	151	121	5	126
		c	151	134	9	143
		d	151	110	14	124
	5.9	a	151	85	71	156
		b	151	59	86	145
		c	151	85	76	161
		d	151	90	70	160
	8.4	a	151	6	148	154
		b	151	12	137	149
		c	151	4	168	172
		d	151	5	134	139
	12.0	a	151	0	171	171
		b	151	0	154	154
		c	151	0	146	146
		d	151	0	157	157
	17.2	a	151	0	157	157
		b	151	0	188	188
		c	151	0	144	144
		d	151	0	114	114
	24.0	a	151	0	88	88
		b	151	0	151	151
		c	151	0	89	89
		d	151	0	96	96

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
NOAA 2	0	a	151	131	17	148
		b	151	158	10	168
		c	151	154	13	167
		d	151	157	18	175
	2.9	a	151	76	104	180
		b	151	145	10	155
		c	151	138	4	142
		d	151	137	16	153
	4.1	a	151	19	115	134
		b	151	138	19	157
		c	151	125	16	141
		d	151	127	14	141
	5.9	a	151	93	53	146
		b	151	112	58	170
		c	151	73	62	135
		d	151	90	86	176
	8.4	a	151	3	156	159
		b	151	13	151	164
		c	151	17	139	156
		d	151	5	146	151
	12.0	a	151	0	165	165
		b	151	0	147	147
		c	151	0	155	155
		d	151	0	*151*	*151*
	17.2	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	24.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*

Italics indicates estimate. When no normal larvae were observed, abnormals were estimated.

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
NOAA 3	0	a	151	141	17	158
		b	151	158	5	163
		c	151	134	20	154
		d	151	122	36	158
	2.9	a	151	122	30	152
		b	151	156	25	181
		c	151	138	40	178
		d	151	142	28	170
	4.1	a	151	158	12	170
		b	151	106	44	150
		c	151	147	32	179
		d	151	129	19	148
	5.9	a	151	83	63	146
		b	151	87	77	164
		c	151	77	93	170
		d	151	79	69	148
	8.4	a	151	12	128	140
		b	151	4	157	161
		c	151	5	153	158
		d	151	4	162	166
	12.0	a	151	0	150	150
		b	151	0	145	145
		c	151	0	160	160
		d	151	2	172	174
	17.2	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	24.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*
	35.0	a	151	0	*151*	*151*
		b	151	0	*151*	*151*
		c	151	0	*151*	*151*
		d	151	0	*151*	*151*

Italics indicates estimate. When no normal larvae were observed, abnormals were estimated.

Station ID	Cu Concentration (ppb)	Rep	Initial # Alive	Final # Normal	# Abnormal	Total
DOC	0	a	151	130	20	150
		b	151	137	28	165
		c	151	115	34	149
		d	151	123	35	158
	2.9	a	151	130	31	161
		b	151	132	27	159
		c	151	156	32	188
		d	151	135	33	168
	4.1	a	151	117	49	166
		b	151	141	18	159
		c	151	141	25	166
		d	151	138	20	158
	5.9	a	151	123	34	157
		b	151	127	27	154
		c	151	113	34	147
		d	151	106	43	149
	8.4	a	151	125	17	142
		b	151	135	29	164
		c	151	104	40	144
		d	151	130	32	162
	12.0	a	151	81	39	120
		b	151	81	53	134
		c	151	129	30	159
		d	151	104	56	160
	17.2	a	151	52	96	148
		b	151	55	91	146
		c	151	58	118	176
		d	151	48	120	168
	24.0	a	151	0	165	165
		b	151	2	161	163
		c	151	3	139	142
		d	151	0	140	140
	35.0	a	151	0	146	146
		b	151	0	165	165
		c	151	0	139	139
		d	151	0	140	140

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14. ABSTRACT

This document describes the results from an assessment of both ambient toxicity and copper bioavailability, using toxicity tests with mussel embryos, in surface waters from Sinclair and Dyes Inlets, adjacent to the Puget Sound Naval Shipyard & Intermediate Maintenance Facility (PSNS&IMF), in Bremerton, Washington.

15. SUBJECT TERMS

Mission Area: Environmental Compliance toxicity assesment mussel embryos water-effect ratios

ambient water quality criteria copper concentration dissolved organic carbon copper complexation capacity

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